ATTORNEY DOCKET NO: CXU-382

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application: Stutzenberger, et al.
Serial No: 11/677,132
Filed: October 1, 2003
Title: Adhesin-specific Nanoparticles And Process For Using Same

Examiner: Maury A. Audet
Group Art Unit: 1654
Confirmation No: 3896
Customer No: 22827
Deposit Account No: 04-1403

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. 1.131

Dear Sir:

Fred J. Stutzenberger, Robert A. Latour, Jr., Ya-Ping Sun and Tzuen-Rong J. Tzeng declare:

1. We are the inventors of the claims of the patent application identified above and the inventors of the subject matter described therein.

2. The above-captioned application was filed October 1, 2003.

3. U.S. Published Patent Application No. 2003/0147966 to Franzen, et al., serial no. 10/192,393 has a publication date of August 7, 2003 with a filing date of July 10, 2002, and claims filing benefit of U.S. Provisional Patent Application Serial no. 60/304,236 with a filing date of July 10, 2001.

4. Prior to July 10, 2001 we had conceived of our invention as described and claimed in the subject application with specific regard to methods and materials for agglomerating biologically active microorganisms by use of particles including a hydrophobic polymeric core, a hydrophilic linking agent bound to the core, and a biofunctional material bound to the hydrophilic linking agent. Such conception took place in this country.

5. We were duly diligent following the conception of the invention in that experimentation was carried out and a patent application was filed. Evidence of which is provided in the attached Appendices including:

Appendix A – Initial experimental results dated October 13, 1999 illustrating a reduction in colony-forming units as a function of nanoparticle concentration.

Appendix B – Initial experimental results dated November 10, 1999 illustrating a reduction in *Campylobacter jejuni* colony-forming units as a function of nanoparticle concentration.

Appendix C – Clemson University Confidential Invention Disclosure Form describing inventions of the pending claims and stating that first conception of the invention occurred on or about May, 2000.

Appendix D – Proposal made to the United States Department of Agriculture Cooperative State Research, Education , and Extension Service requesting funding of research related to the inventions of the pending claims dated September 6, 2000. (Dollar amounts have been redacted from the proposal.)

Appendix E – Clemson University memorandum dated November 20, 2000 relating to the procedures necessary for obtaining patent protection of the inventions of the pending claims.

Appendix F – Research Protocol from the Department of Poultry Science of North Carolina State University having an approval date of December 7, 2000.

Appendix G – Memo dated January 3, 2001 regarding the budgeting of the USDA award. (Dollar amounts have been redacted from the memo.)

Appendix H – Annual Report dated August 29, 2001 summarizing research work carried out over the period from the middle of October, 2000 to the date of the report.

Appendix I – Memo dated July 10, 2002 summarizing a patentability opinion on present subject matter.

6. We acknowledge all statements made of our own knowledge in this declaration to be true and all statements made on information and belief in this declaration are believed to be true.

7. We further acknowledge that willful false statements and the like made in this declaration are punishable by fine or imprisonment, or both and may jeopardize the validity of the application or any patent issuing thereon.

1/21/09
Date

Fred Stutzenberger
Fred J. Stutzenberger

Date

Robert A. Latour, Jr.

Date

Ya-Ping Sun

Date

Tzuen-Rong J. Tzeng

7. We further acknowledge that willful false statements and the like made in this declaration are punishable by fine or imprisonment, or both and may jeopardize the validity of the application or any patent issuing thereon.

Date

Fred J. Stutzenberger

Jan. 21, 2009

Date

Robert A. Latour, Jr.

Date

Ya-Ping Sun

Date

Tzuen-Rong J. Tzeng

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Date	Fred J. Stutzenberger
Date	Robert A. Latour, Jr.
1/27/2009 Date	Ya-Ping Sun
Date	Tzuen-Rong J. Tzeng

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Date

Fred J. Stutzenberger

Date

Robert A. Latour, Jr.

Date

Ya-Ping Sun

1-29-09

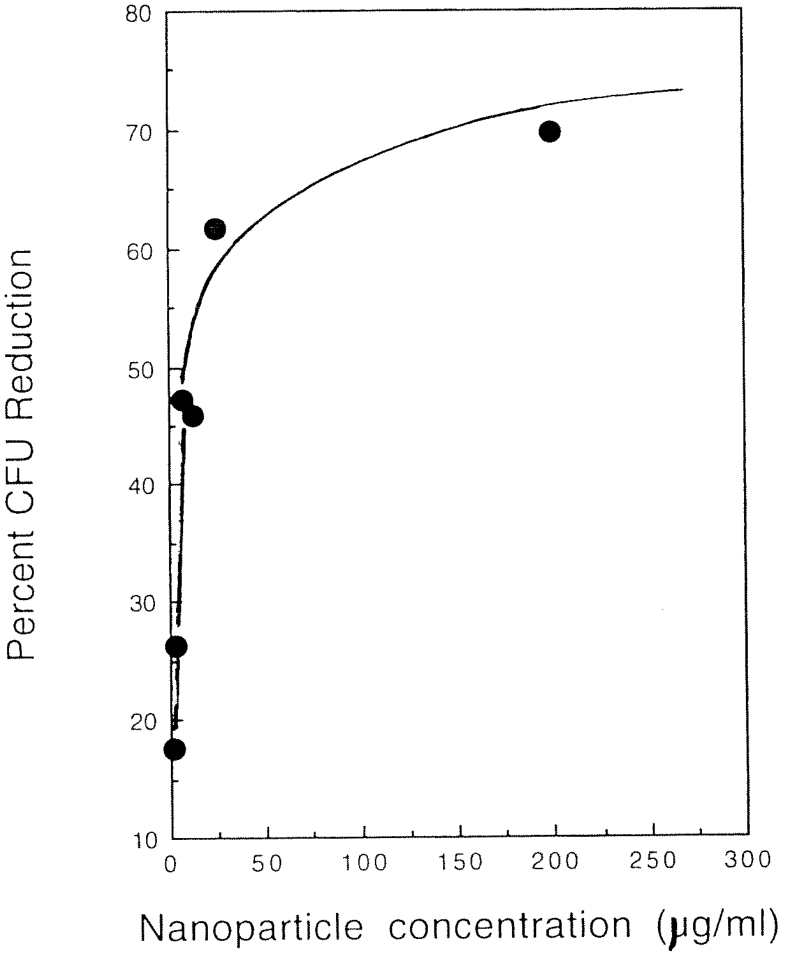
Date

Tzuen-Rong Tzeng

Tzuen-Rong J. Tzeng

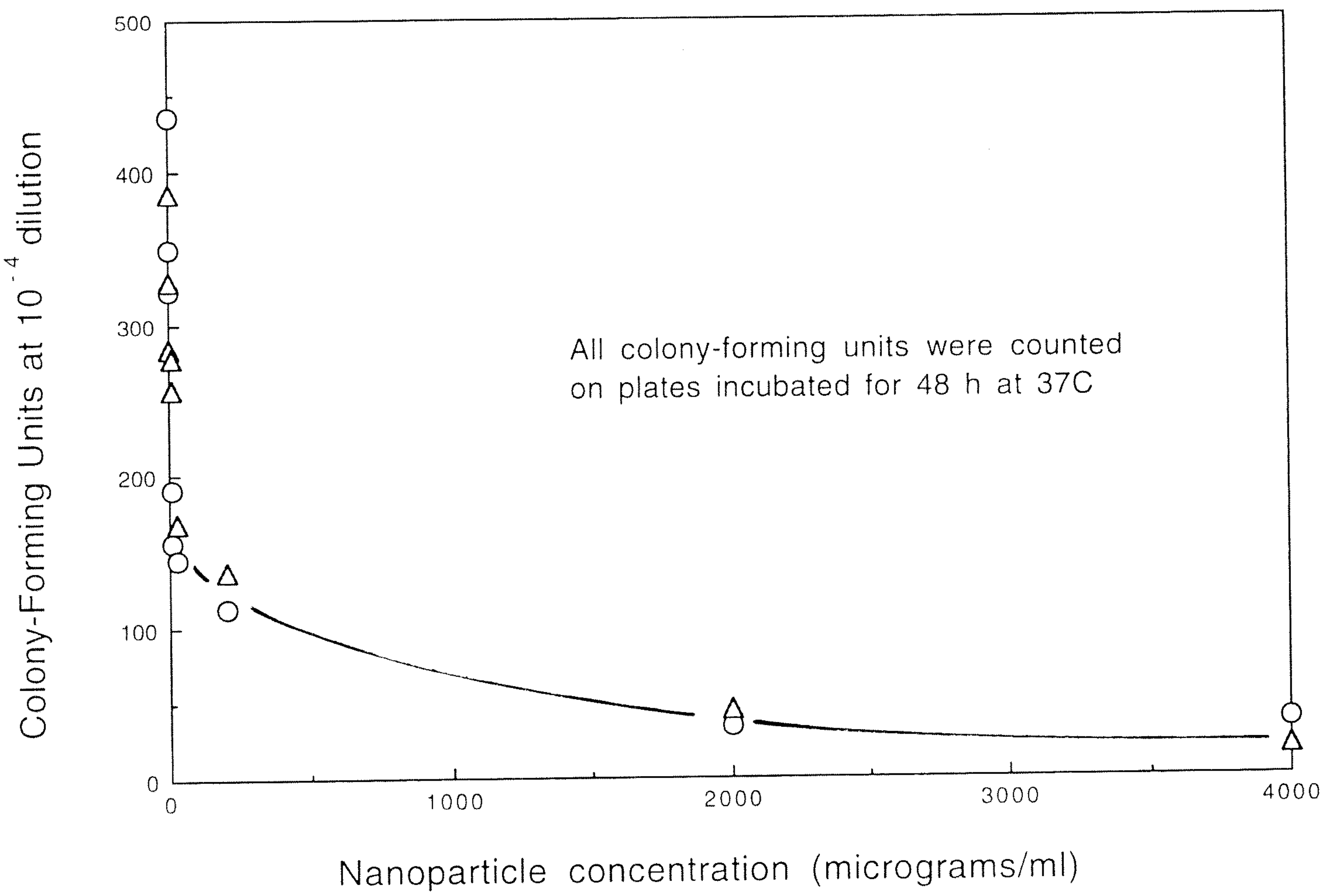
APPENDIX A

10/13/99


Reduction in Colony-forming Units (CFU)
as a function of nanoparticle concentration

APPENDIX B

11/10/99



All colony-forming units were counted on plates incubated for 48 h at 37C

Decrease in Campylobacter jejuni colony-forming units as a function of nanoparticle concentration

APPENDIX C

Clemson University
Confidential Invention Disclosure Form

This form must be completed in its entirety. If any question is not applicable, indicate N/A.

Title of Invention: Adhesin-Specific Nanoparticles

1. Write a brief description of the invention (include keywords to be used in categorizing this invention):

Nanoparticles are synthesized in various controlled size ranges (250 nanometers to 750 nanometers) and functionalized with selected bioactive surface groups. The hydrophobic core of the nanoparticle consists of a polystyrene polymer to which functionalized polyethylene glycol tether are covalently attached. A variety of hydrophilic bioactive functional groups (such as sialic acid, mannose and various polypeptides which are present on the surfaces of human cells) are covalently attached to the polyethylene glycol tethers. These bioactive groups function in vivo as attachment points on human cell surfaces to which pathogenic bacteria naturally adhere via their surface adhesins. Since nanoparticles have the same bioactive groups on their surfaces, they are able to specifically aggregate pathogenic bacteria having complementary adhesin sites on their surfaces. Aggregation by nanoparticles will inhibit their binding to host cells by sterically blocking their adhesins to prevent the adherence phase of the normal infection/colonization sequence.

2. When did you first conceive of the invention? On or about May, 2000

3. What is the stage of development?

____ **Working prototype**

X **Proof of concept**

____ **Analytical work**

4. Has concept been reduced to practice? If not, what further effort is required?

The concept has not yet been put into practice. The analytical steps which are required are:

a. testing the bacteria-aggregation potential in vitro using targeted bacteria which have known adhesin affinities for the bioactive groups on the nanoparticles.

b. Testing of the nanoparticles in an animal model to evaluate their ability to purge specific bacteria from the intestinal tract

c. Determination of any potential toxicity by the nanoparticles to the host using standard animal models.

5. Was the work that led to the Invention sponsored by industry or funded by State or Federal appropriations? If so, please attach a copy of the contract or agreement, and complete the following blanks:

No, not at this time.

a) **Name of Government agency:** ________________

Contract or Grant # and Date:

Name & Address of Grants Officer:

b) Name of Industrial Company:

c) Name of Private Sponsor:

d) State or Federal Appropriation:

6. If the Invention has or will be described orally or in writing to someone skilled in the art, or in trade journals or publications, please list titles, dates, etc. and attach copies, if possible.

None

7. Which of the following have you done or do you intend to do?

	YES	**NO**	**DATE**
• **Publish**	X		
• **Oral Presentation**	X		
• **Poster Session**	X		
• **Disclose to Industry Rep**		X	
• **Other Public Dissemination**	X		

8. Please write a brief, non-confidential abstract describing your Invention. This will be posted on the webpage (http://hubcap.clemson.edu/IPC/) and used for marketing purposes. If you have any objections to posting on the webpage, please explain.

Project Summary

Campylobacter species (campylobacters) are ubiquitous, Gram-negative bacteria which cause abdominal cramps and profuse bloody diarrhea in humans. Large case-control studies have shown that 50-70% of campylobacter infections can be traced to poultry meat products. The overall goal of this proposal is to develop and evaluate a novel, farm-based strategy for the removal of campylobacters from poultry intestinal tracts. This strategy will employ specifically bioactive nanoparticles which bind to C. jejuni surfaces. These bioactive nanoparticles will be orally administered to chickens and turkeys; the aim is to dislodge colonized campylobacters from the intestinal lining and facilitate fecal expulsion of campylobacter/nanoparticle aggregates. Objectives include: 1) synthesis of nanoparticles consisting of a hydrophobic polymer core with a surface array of covalently linked host cell receptor molecules having a high affinity for campylobacter adhesins, 2) in vitro screening of various nanoparticle compositions for their ability to agglutinate pure cultures of C. jejuni as well as selected other poultry-associated enteropathogens, 3) evaluation of the most agglutinative nanoparticle compositions as C. jejuni purging agents in chickens and turkeys, 4) determination of the nanoparticle toxicity potential in mice and rabbits. This proposal encompasses elements of research, education and extension which relate to the National Food Safety Initiative goal of developing control measures for food-borne microbial pathogens.

9. What are the novel and unique features of your Invention? Cite current technology and describe how Invention differs? What are its advantages?

Current technology for the treatment of gastrointestinal infections (whether in animals or humans) involves the prolonged use of antibiotics. However, the use of antibiotics creates the potential for adverse side effects such as allergic reactions and unfavorable alteration of normal intestinal flora. Moreover, many bacterial species are now resistant to a wide range of clinically prevalent antibiotics. The widespread use of antibiotics in agricultural animals (such as cattle and poultry) has encouraged the emergence of antibiotic-resistant bacterial strains. Therefore, antibiotic treatment of intestinal bacterial infections is gradually diminishing in its effectiveness.

Pathogenic bacteria bind to human cells via specific surface sites termed adhesins. These bacterial adhesins have affinity for specific sugars and peptides which are normal components of the human cell surface. Nanoparticles which carry the same sugars or peptides will therefore be adhesin-specific, enabling them to bind to the bacterial surface, aggregate the bacteria and prevent them from attaching to the human cells. The advantages of using nanoparticles rather than antibiotics are:

a. Nanoparticles are adhesin-specific so they will not bind to other intestinal flora which lack that specific adhesin.

b. Unlike mutation to antibiotic resistance, mutation to nanoparticle non-adherence will eliminate a virulence factor essential to the infection/colonization process

c. Nanoparticle specificity can be easily altered to aggregate any microbial pathogen (bacterial, fungal, viral, parasitic) which carries a defined surface adhesin; therefore, nanoparticle therapy has the potential of being much more versatile than the antibiotics currently in use.

d. Since nanoparticles are composed of hydrophobic polystyrene cores surrounded by bioactive surface groups which are normal body components, the potential for the development of adverse side effects is greatly diminished.

10. What are the possible immediate and future commercial applications for the Invention?

List one or more specific applications.

a. Nanoparticles could be used as a low cost, specific purging agent for the removal of Campylobacter jejuni (the most common poultry-associated human pathogen)from chickens and turkeys prior to processing for market.

b. Nanoparticles could be used as therapeutic agents to cure or reduce chronic bacterial, fungal or parasitic infections in the human digestive tract.

c. The application of nanoparticles as anti-biowarfare agents has great potential as surface decontaminants.

d. Nanoparticles could be clinically employed as a diagnostic tool for the identification of pathogenic microorganisms having specific adhesins (i.e. agglutination test).

Inventor #1

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Singature - Inventor #2

Inventor #3

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Inventor #4

Tzuen-Rong	J	Tzeng
First Name	*Middle Initial*	*Last Name*
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Tzuen-Rong Tzeng

Singature - Inventor #4

APPENDIX D

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

ASSURANCE STATEMENT(S)

OMB Approved 0524-0022
Expires 5/31/98

TATEMENT OF POLICY - Safeguarding the rights and welfare of subjects at risk ld the proper isolation security of research agents in activities supported by ooperative State Research, Education, and Extension Service is the responsibility the institution to which support is provided. In order to provide for the adequate discharge of this responsibility, USDA policy requires a formal assurance that appropriate committees in each Institution will carry out both initial review of proposals and continuing review of supported projects. The Department also requires certification of such reviews.

OTE: Check appropriate statements, supplying additional information when necessary

INSTITUTION

CLEMSON UNIVERSITY

2. TYPE

☒**New** ☐**Extension** ☐**Revision**

3. CSREES PROJECT NUMBER OR AWARD NUMBER (If Known)

TITLE OF PROJECT

Adhesin-Specific Nanoparticles for Removal of *Campylobacter jejuni in* Poultry

5. PRINCIPAL INVESTIGATOR(S)

Robert A. Latour
Fred J. Stutzenberger

. RECOMBINANT DNA OR RNA RESEARCH

☒ **Project does not involve recombinant DNA or RNA.**
☐ **Project involves recombinant DNA or RNA** (Check the applicable statement).
  ☐ This project has been determined by the local IBC to be exempt from the NIH Guidelines.
  ☐ This project is under review by the local IBC and a revised Form CSREES-662 or an approval letter signed by Chair, IBC will be submitted when the review is completed.
  ☐ This project has been reviewed by an IBC and was approved on ____________(Date).

This institution agrees to assume primary responsibility for complying with both the intent and procedures of the National Institutes of Health's (NIH) "Guidelines for Research Involving Recombinant DNA Molecules," as revised (see subsection 205(b)(3), Subpart U of the "Uniform Federal Assistance Regulations" (7 CFR Part 3015) and other applicable Federal/State guidelines and regulations.

This responsibility includes:

1. Ensuring that a standing Institutional Biosafty Committee (IBC) reviews proposed projects.
2. Registering with the IBC all experiments involving recombinant DNA and RNA Molecules conducted with the funds provided under this project/grant and complying with the requirements specified in Part II of the NIH Guidelines or any other pertinent guidelines and regulations. IBC's are required to keep records of this research in a form that is available to the U.S. Department of Agriculture (USDA) upon request.

In addition, principal investigators must report the following to the USDA and to their IBC's:

1. New Technical information relating to risks and safety procedures.
2. Serious accidents or releases involving recombinant DNA or RNA.
3. Serious illness of a laboratory worker which may be project related.
4. Other safety problems.

. ANIMAL CARE

☐ **Project does not involve use of vertebrate animals.**
☒ **Project involves use of vertebrate animals.** (Check the applicable statement).
  ☒ a) The project is in compliance with the Animal Welfare Act of 1966 and 9 CFR Subchapter A (Laboratory Animals), as amended.
  ☐ b) This project is under review by the Institutional Animal Care and Use Committee and a revised Form CSREES-662 or an approval letter signed by Chair of the committee will be submitted when the review is completed.
  ☐ c) This project has been approved by the Institutional Animal Care and Use Committee on ____________(Date).

. PROTECTION OF HUMAN SUBJECTS

☒**Project does not involve use of human subjects.**
☐ **Project Involves use of human subjects.** (Check the applicable statement).
  ☐ a) This project includes activities involving human subjects but can in no way be considered at risk. (If this statement is checked, the person signing this form must also initial in the space at the right.) ____________(Initial)
  ☐ b) This project is under review by an institutional committee as provided by our assurance and a revised Form CSREES-662 or an approval letter signed by Chair of the committee will be submitted when the review is completed.
  ☐ c) This project includes activities involving human subjects. Our institutional committee reviewed and approved it on ______________in accordance with our assurance approved by USDA and/or DHHS. The project will be subject to continuing review as provided for in that assurance.

IGNATURE OF AUTHORIZED ORGANIZATIONAL REPRESENTATIVE for Y. T. Shah	**TITLE** Chief Research Officer and Senior Vice Provost	**DATE** 9-06-00

orm CSREES-662 (6/95)

LATOUR

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3. What is the stage of development?

____ **Working prototype**
_X_ **Proof of concept**
____ **Analytical work**

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5. Was the work that led to the Invention sponsored by industry or funded by State or Federal appropriations? If so, please attach a copy of the contract or agreement, and complete the following blanks:

No, not at this time.

a) **Name of Government agency:** ____________________

Contract or Grant # and Date: ______________________

Name & Address of Grants Officer: ______________________

b) Name of Industrial Company: ______________________

c) Name of Private Sponsor: ______________________

d) State or Federal Appropriation: ______________________

6. If the Invention has or will be described orally or in writing to someone skilled in the art, or in trade journals or publications, please list titles, dates, etc. and attach copies, if possible.

None

7. Which of the following have you done or do you intend to do?

	YES	NO	DATE
• **Publish**	X		
• **Oral Presentation**	X		
• **Poster Session**	X		
• **Disclose to Industry Rep**		X	
• **Other Public Dissemination**	X		

8. Please write a brief, non-confidential abstract describing your Invention. This will be posted on the webpage (http://hubcap.clemson.edu/IPC/) and used for marketing purposes. If you have any objections to posting on the webpage, please explain.

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c. Nanoparticle specificity can be easily altered to aggregate any microbial pathogen (bacterial, fungal, viral, parasitic) which carries a defined surface adhesin; therefore, nanoparticle therapy has the potential of being much more versatile than the antibiotics currently in use.

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b. Nanoparticles could be used as therapeutic agents to cure or reduce chronic bacterial, fungal or parasitic infections in the human digestive tract.

c. The application of nanoparticles as anti-biowarfare agents has great potential as surface decontaminants.

d. Nanoparticles could be clinically employed as a diagnostic tool for the identification of pathogenic microorganisms having specific adhesins (i.e. agglutination test).

Inventor #1

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Singature - Inventor #2		

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Singature - Inventor #3		

Inventor #4

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Home Address		*Home Phone*
Tzuen-Rong Tzeng		
Singature - Inventor #4		

[illegible]te for [illegible] PI
6/5/00

Name/phone for proposal pick-up

Dianne [illegible]

CLEMSON UNIVERSITY
Sponsored Programs Processing Form

Office for Sponsored Programs: 864-656-2424

For Internal Use Only

01274
OSP Number

College Use

PRINCIPAL INVESTIGATOR/CO-INVESTIGATORS - *LAST NAME FIRST* (FOR ADDITIONAL SPACES, USE SUPPLEMENT #1)

Principal Investigator/Co-Investigators (List Principal Investigator First)	Department Number to be credited with project	University I.D. (email)	Credit to Project (Must Total 100%)
Robert A. Latour	0905	latourr	27.5%
Fred J. Stutzenberger	0327	sfred	27.5%
Ya-Ping Sun	0973	syaping	20%

[illegible]AL PROPOSAL [illegible]

IF APPLICABLE, unit number of **Center/Institute** affiliated with or responsible for project -- *Center Director signature required on reverse.*

TITLE **Adhesin-Specific Nanoparticles for Removal of Campylobacter jejuni from Poultry**

Period Covered From: 0 1 0 1 2 0 0 1 To: 1 2 3 1 2 0 0 3

FUNDING AGENCY USDA

Sponsor Type:
☒ Federal ○ Federal thru SC State Agency ○ State of South Carolina ○ Industry ○ Foundation ○ Other

Classification:
○ Instruction ☒ Research ○ Public Service ○ Regulatory ○ Other

OSP USE: 201

Foreign Sponsor?
○ Yes ☒ No

Submission Type
☒ New ○ Change, provide project # or proposal #

REQUESTED FUNDS

Direct Costs Requested	F&A (Indirect) Costs Requested	TOTAL REQUESTED FUNDS
[redacted]	[redacted]	[redacted]

Check box if cost sharing is proposed. A fully approved **COST SHARE AGREEMENT (Form OSP-1A)** must be attached. ☐

COLLEGE USE (IF REQUIRED)

○ Overnight
○ Certified
○ Fax
○ Return to PJ

Complete address, fax # or special instructions

In

Out

SPONSORED PROGRAMS USE ONLY

Sponsor Restricted Indirect Cost Rate

F&A (I.C.) rate allowed by sponsor .23456

Unrecovered F&A (I.C.) [redacted]

Indirect on Associated Direct Cost Shared ($)

Date In [illegible]

Date Out [illegible]

CFDA

Staff Use (Grants Coordinator initial)
PC 6-2-00
et 6-6-00

Sponsor Code
207

OSP-1
Rev. July

Duplicate as needed

CLEMSON UNIVERSITY
Sponsored Programs Processing Form

SUPPLEMENT #1

OSP Number

College Use

ADDITIONAL CO-INVESTIGATORS - *LAST NAME FIRST*

Co-Investigators	Department Number (to be credited)	University I.D. (email)	Credit to Project (Must Total 100%)
John Rodgers	0366	jrodger	10%
Tzuen-Rong Tzeng	0327	tzuenrt	15%

ADDITIONAL CERTIFICATION AND APPROVAL SIGNATURES

Financial Disclosure: The undersigned certify that they have read, understand, and are bound by Clemson University's FINANCIAL DISCLOSURE policy, tha they have made all financial disclosures required by it, if any, and will comply with any conditions or restrictions imposed by the institution to manage, reduce, c eliminate actual or potential **conflicts of interest.** Intellectual Property: The undersigned certify that they have read, understand, and are bound by Clemso University's patent policy and computer software copyright policy, agree to assign all rights, title, and interest in intellectual property under such policies t Clemson University, and to execute such further documents as needed to perfect the assignment of such rights. All investigators agree to disclose, and to caus other project personnel to disclose, all INTELLECTUAL PROPERTY to the University Intellectual Property Committee within 60 days of discovery or the time o confidential submission for publication of manuscripts disclosing the invention, whichever is earlier. Failure to make timely disclosure to the Committee may lea to the loss of patent rights to the federal government on federal government contracts or to other parties. Cost AccountingStandards: The principal investigato certifies that the proposed budget complies with Clemson University's federal cost accounting standards policy.

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Adhesin-Specific Nanoparticles for Removal of *Campylobacter jejuni* from Poultry

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Adhesin-Specific Nanoparticles for Removal of *Campylobacter jejuni* from Poultry

Project Summary

Campylobacter species (campylobacters) are ubiquitous, Gram-negative bacteria which cause abdominal cramps and profuse bloody diarrhea in humans. Large case-control studies have shown that 50-70% of campylobacter infections can be traced to poultry meat products. The overall goal of this proposal is to develop and evaluate a novel, farm-based strategy for the removal of campylobacters from poultry intestinal tracts. This strategy will employ specifically bioactive nanoparticles which bind to *C. jejuni* surfaces. These bioactive nanoparticles will be orally administered to chickens and turkeys; the aim is to dislodge colonized campylobacters from the intestinal lining and facilitate fecal expulsion of campylobacter/nanoparticle aggregates. Objectives include: 1) synthesis of nanoparticles consisting of a hydrophobic polymer core with a surface array of covalently linked host cell receptor molecules having a high affinity for campylobacter adhesins, 2) *in vitro* screening of various nanoparticle compositions for their ability to agglutinate pure cultures of *C. jejuni* as well as selected other poultry-associated enteropathogens, 3) evaluation of the most agglutinative nanoparticle compositions as *C. jejuni* purging agents in chickens and turkeys, 4) determination of the nanoparticle exposure sensitivity levels using rat and rabbit models to address human health risk potential associated with nanoparticle handling and use. This proposal encompasses elements of research, education and extension which relate to the National Food Safety Initiative goal of developing control measures for food-borne microbial pathogens.

Adhesin-Specific Nanoparticles for Removal of *Campylobacter jejuni* from Poultry

I. PROJECT DESCRIPTION

I.1. JUSTIFICATION AND LITERATURE REVIEW

I.1.a. Program Area Addressed by this Proposal

This proposal describes a novel approach for the control of food-borne microbial pathogens (Program Area 111.2) in commercial poultry at the farm level prior to transport and processing. Although *Campylobacter jejuni* strain Epi23 (a poultry isolate obtained from USDA) has been chosen as the primary targeted model enteropathogen against which to test the process capability of this approach, other food-borne enteropathogens (*Salmonella* and *Escherichia spp.*) will also be included as targets.

I.1.b. Magnitude of food safety problem

Campylobacter species (campylobacters) are curved, spiral-shaped or gullwing-shaped motile Gram (-) bacilli. Campylobacters are considered to be naturally occurring members of the gastrointestinal flora of birds and mammals, and have been associated with diarrhea in agricultural livestock [1]. The first reported outbreak of campylobacter enteritis in humans occurred in 1978 and involved an estimated 3,000 people [2]. As campylobacter isolation techniques became available and the clinical awareness of their pathogenesis increased, it became clear that these bacteria were a major cause of diarrheal illness. By the early 1990's, campylobacters had replaced *Salmonella sp.* as the most common cause of food-borne diarrhea in the western world [3].

Although there are at least 14 *Campylobacter* species, *C. jejuni* has been identified as the causative agent in 99% of the reported cases of diarrheal illness in which isolation and identification were made [4]. The infectious dose of *C. jejuni* in humans is relatively low (~500 viable cells, [5]) compared to other enteric pathogens. Following an incubation period of 2-10 days during which the campylobacter colonizes the mucosa of the small intestine, the symptoms generally include abdominal pain, fever and watery, bloody stools). Symptoms are generally more severe in the populations of developed countries than in those of the developing world, presumably because the latter have acquired partial immunity [6].

Commercial poultry is currently the chief source of *C. jejuni* infection in humans. Commonly, more than half the poultry carcasses worldwide are found contaminated at point of sale with *C. jejuni*. [7-9]. The birds normally harbor many campylobacters in the cecum, a commensal colonization which can occur during early growth [10]. However, a continuous source outbreak has been traced to a single farm growing chickens colonized with a single *C. jejuni* serotype [11].

Spread of campylobacters among hatch mates is rapid if infected birds are introduced into the population; under laboratory conditions, three days of contact with artificially inoculated seeder birds is sufficient for the majority of the brood to be colonized [12]. Young chicks are highly susceptible to campylobacter colonization (minimal infective dose for 50% of a 24 hour old population is 35 colony forming units, [13]). Chickens are coprophagic, which facilitates the fecal-oral spread of campylobacters; however, the rapidity of the shift from uncolonized to 100% colonized suggests that campylobacters are also spread from chick to chick via their communal source of drinking water [14]. Campylobacter transmission via water again becomes important during mass-mechanized processing for market, since uncontaminated birds become contaminated via water used in the defeathering or chilling processes. Processing plant contamination is exacerbated by the cooping and transport of birds. Campylobacter populations on the feathers of cooped and transported birds are >10 fold greater than those of remaining brood mates [15]. This combination of factors constitutes a major mechanism for processing plant contamination [16].

At the consumer level, accidental ingestion of one drop of raw chicken juice can easily constitute an infectious dose [4]. Infections can occur during the improper handling of raw chicken carcasses, by eating insufficiently cooked chicken (still bloody and pink near the bone), and via cross-contamination of other foods by contact with knives or cutting boards used to prepare chicken [17].

The problem of campylobacters in the food chain must be effectively addressed, but to date there are no practical and effective measures available [18]. While some reduction in cross-contamination of poultry carcasses can be achieved by improved sanitation during processing, the elimination of campylobacter populations from birds *prior* to processing is obviously preferable [19]. Various strategies have been advanced to reduce the rates of poultry contamination: introduction of competing microbial populations into newly hatched chicks, chlorination of poultry drinking water, vaccination or selective breeding of poultry for resistance to campylobacter colonization (reviewed, [18]).

Since the most important source of campylobacter contamination of poultry is the farm [16], the farm is the location at which that contamination should be controlled. Toward that goal, we propose the development of polymeric based nanoparticles, the surface of which carry molecules that are known host cell receptors for bacterial adhesins. These host cell receptors (fibronectin fragments, D-mannose, sialic acid) are moities of extracellular glycoproteins which bind to fimbrial adhesin sites of several enteric pathogens and sterically hinder their function [20]. The favorable potential of this approach has been suggested by an earlier study [21] in which *Salmonella typhimurium* colonization was markedly reduced in the caecum of broiler chickens by feeding high concentrations of mannose. If these sites can be blocked by nanoparticles bearing host cell receptor molecules (such as mannose and the others listed above), the adherence of enteric pathogens to intestinal mucosal epithelium will be inhibited as has already been shown for soluble mannose [22]. The ability of these bioactive nanoparticles to adhere to campylobacter surfaces will enable them to compete with host cell receptors to reduce or eliminate the extent of campylobacter colonization on the poultry intestinal wall. Therefore these nanoparticles, which are readily synthesized in a wide range of sizes and receptor selection, will be evaluated as purging agents to remove campylobacters and several other human enteropathogens from the intestinal contents of commercial poultry (chickens and turkeys) prior to transport and processing.

I.2. OBJECTIVES

I.2.a. Specific Objectives of the Proposed Research

The **short term objectives** of the proposed study are:

1. To design and synthesize nanoparticles of controlled size distribution with surface affinities for specific sites on bacterial surfaces. These nanoparticles will consist of hydrophobic core polymer chains covalently linked to molecules that are known host ligands for surface adhesins of food-borne enteropathogens.

2. To test the *in vitro* potential of these synthesized nanoparticles to aggregate pure cultures of *Campylobacter jejuni, Salmonella enteritidis* and *Escherichia coli.* These bacteria are recognized human enteropathogens widely associated with commercial poultry products.

3. To demonstrate whether nanoparticle specificities that have high aggregative activity under controlled, pure culture conditions can also be effective agents to purge enteropathogens from the complex intestinal environment in chickens and turkeys. Selected nanoparticles will be tested in birds from hatch to harvest to determine the efficacy and duration of their purgative effect.

4. To investigate health and safety aspects of human exposure to the bioactive nanoparticles via in vitro cell culture and *in vivo* ingestion, inhalation, skin, and ocular testing using rat and rabbit models.

5. To introduce food safety issues in graduate student educational programs by direct involvement of graduate students with this state-of-the-art food safety technology program. In addition, graduate students involved with this program will present oral research reports on this program in their graduate seminar classes to their respective graduate student body within the departments of Bioengineering, Chemistry, and Microbiology & Molecular Medicine at Clemson University, as well as the faculty and graduate students participating in the Greenville Hospital System/Clemson University Biomedical Cooperative. This will provide an excellent mechanism to increase awareness of food-borne pathogen problems and issues.

6. To demonstrate the importance and potential benefit of university extension service by conducting the proposed program at the actual farm level of practice. If successful, this program will thus have obvious and direct practical application and benefit for food safety at the production source.

The **long term objective** of this overall program is to demonstrate the ability of a synthesized, adhesin-specific nanoparticle to bind to, form aggregates with, and reduce the infective capability of human food-borne enteropathogens. While commercial poultry are proposed as the model system for its debute, it is conceivable that the nanoparticle strategy may prove useful in numerous other food applications for beef, pork, fruits, and vegetables such as an antibiotic-free food decontamination agent where the ability to sterically inhibit the adherance of pathogens to host surfaces will prevent or substantially reduce the infective process.

I.3. METHODOLOGY

I.3.i. PROGRAM OVERVIEW

The proposed program will be undertaken in four inter-related conmpo: In the first part, nanoparticles will be synthesized in various controlled size ranges and functionalized with selected surface functional groups. In the second part, the bioactivity of the synthesized nanoparticles will be assessed using in vitro cell culture studies with selected target microbial pathogens. The most effective nanoparticles from this study will then be used in in vivo farm-based bioactivity studies and in both in vitro and in vivo exposure sensitivity tests to evaluate the safety of handling and use of these nanoparticle systems. The specific details for each of these four parts are discussed in the following sections.

I.3.ii. MATERIALS & METHODS: PART 1. BIOACTIVE NANOPARTICLE SYNTHESIS

As stated above, the objective of this component of the program is to synthesize nanoparticles in controlled particle size ranges with the designated surface functionality selected for its potential adhesiveness to adhesin molecules presented by *Campylobacter jejuni.*

I.3.ii.a. Nanoparticles for Biological Decontamination/Bacterial Treatment

The overall concept for the proposed program is based on the preparation, application, and evaluation of nanoscopic particles that are chemically functionalized with bioactive species or bacterial adhesins selected for their potential for high-affinity binding to targeted microbial pathogens.

I.3.ii.b. Nanoparticle Systems

Two kinds of nanoparticle systems will be employed. One is based on the self-assembly of organic polymers structured with intrapolymer binary pseudo-phase separation character. Here, self-assembly is driven by the experimental conditions for micellar formation [23,24]. Average sizes of the nanoparticles will be adjusted by controlling the micellar formation conditions. Upon the formation of the nanoparticles, a photochemical curing process will be applied in order to produce intra-particle cross-links, and thus "lock" in the nanoparticle structure to the formed shapes, sizes, and morphology (illustrated in Figure 1 below).

While there is a wide selection of polymers for the task, we will begin with those that are structurally simple and inexpensive, such as the para-functionalized polystyrene copolymers. Here, the hydrophilic para-functionality will facilitate the intrapolymer pseudo-phase separation; and it will also be used as a linkage for the adhesin attachment. Accordingly, polystyrene polymers para-finctionalized with polyethylene glycol (PEG) are illustrated in Scheme 2. The copolymer composition can be adjusted for controlling nanoparticle sizes via intrapolymer pseudo-phase separation. The end carboxylate protection can be removed either completely or partially via hydrolysis, which allows some control in the loading of designated bioactive adhesins.

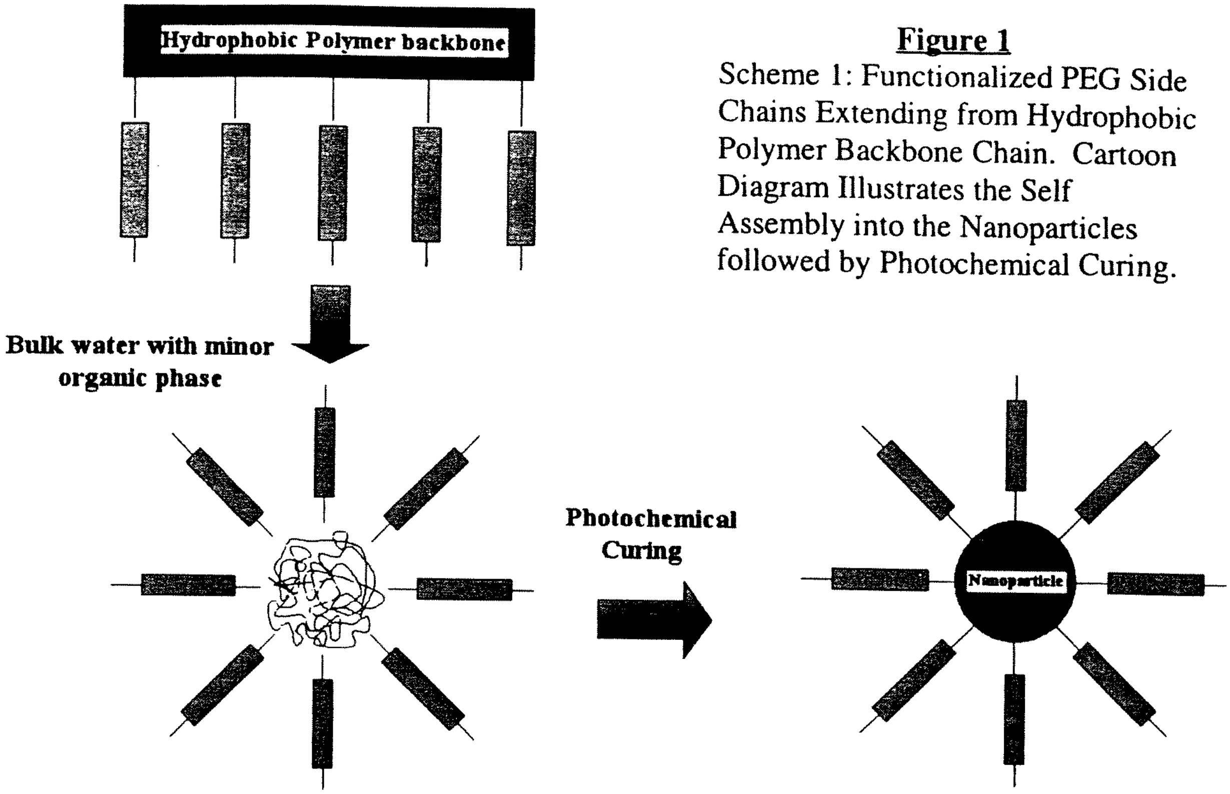
Hydrophobic Polymer backbone
Bulk water with minor organic phase
Photochemical Curing
Nanoparticle

Figure 1

Scheme 1: Functionalized PEG Side Chains Extending from Hydrophobic Polymer Backbone Chain. Cartoon Diagram Illustrates the Self Assembly into the Nanoparticles followed by Photochemical Curing.

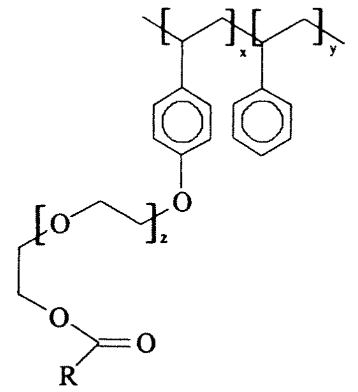

Figure 2

Scheme 2. Functionalized PEG Branches (R) Tethered to Polystyrene Backbone Chain

The other nanoparticle system is based on the organic chemical functionalization of inorganic nanoparticles. The nanoparticles will be produced via applications of supercritical fluid processing techniques [25-27]. Among our first choices are oxide nanoparticles, such as TiO_2 and ZnO, for which the particle surface coating by organic species are well established. As illustrated in Scheme 3, the same PEG linkages can be used for the adhesin attachment and for the control of adhesin loading in the nanoparticle system.

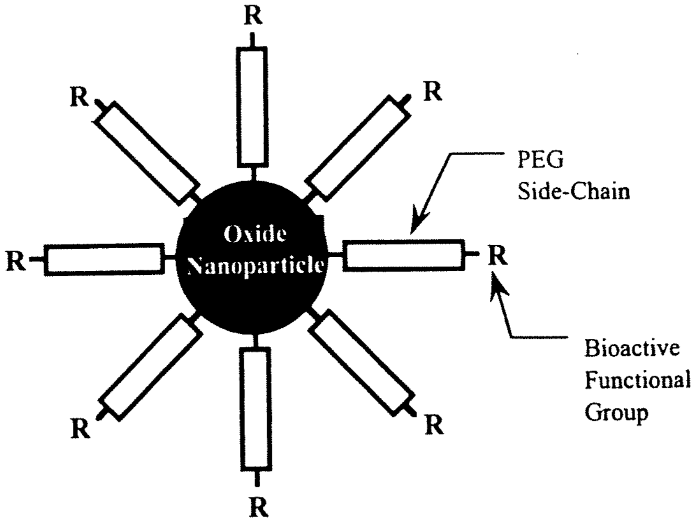

Figure 3
Scheme 3: Oxide Core Nanoparticle with
PEG Tethered Bioactive Functional Groups

I.3.ii.c. Bioactive Molecules for Nanoparticle Surface Functionalization

The potential bioactive molecules under consideration include sialic acid, D-mannose, bioactive polypeptide sequence #1 (Sigma-A5582, N-Acetyl-Pen-Arg-Gly-Asp-Cys with disulfide linkage between Pen and Cys thus providing RGD sequence in ring structure; shown to be a fibrinogen receptor antagonist [28,29]; see Figure 5), bioactive polypeptide sequence #2 (Sigma-A6677, Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro which provides a 10 residue peptide straight chain containing RGD shown to inhibit fibroblast binding to fibronectin [30-32]), and bioactive polypeptide sequence #3 (Sigma-G5646, Gly-Arg-Gly-Asp-Thr-Pro which provides an RGD containing 6 residue peptide straight chain shown to inhibit cell attachment to fibronectin, vitronectin, and Type I collagen [33-35]). All of these molecules and molecular segments are components of the extracellular matrix glycoproteins which are reported in the literature to represent the specific binding sites for microbial pathogens, such as *Campylobacter jejuni*, *Salmonella*, and *Escherichia* spp [36]. By presenting these same functional groups from the surface of the nanoparticles, the nanoparticles should effectively compete with the mucusal lining of the gastrointestinal tract for microbial attachment. Furthermore, because the nanoparticles will contain a relatively large surface density of functional sites, they should be able to agglutinate large numbers of pathogens, prevent them from binding to their poultry host, and enable them to be safely purged from the gastrointestinal system. The molecular structures of each of these potentially bioactive molecules is shown in Figures 4-7 below.

Each of these bioactive functional groups will be covalently linked to the end of PEG tether molecules extending from the core hydrophobic polymer chain or metal oxide. Coupling will be accomplished via appropriate chemistry routes with either an OH group on the sialic and D-mannose, the N-acetyl terminus for bioactive peptide sequence #1, or the carboxyl terminus of the bioactive polypeptide chains #2 and #3. Specific care will be taken in the chemical synthesis routes to protect RGD peptide sequence functionality.

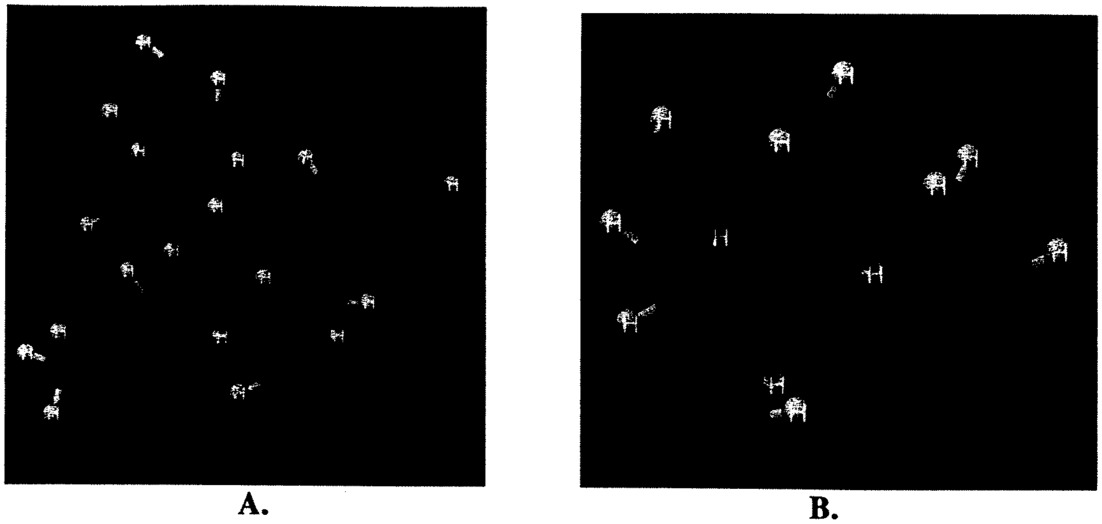
A. B.

Figure 4

Molecular Structures of Sialic Acid (A), and D-Mannose (B)
Color Code: Grey = C, White = H, Red =O, Blue = N

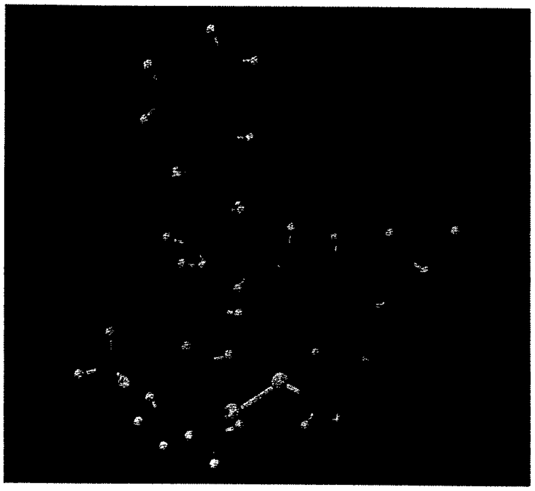

Figure 5

Molecular Structures of an RGD Containing Bioactive Polypeptide Sequence #1 (Sigma A5582)
N-Acety-Pen-Arg-Gly-Asp-Cys (or N-Acetyl-Pen-RGD-C); disulfide bond between Pen & Cys.
Grey = C, White = H, Red =O, Blue = N

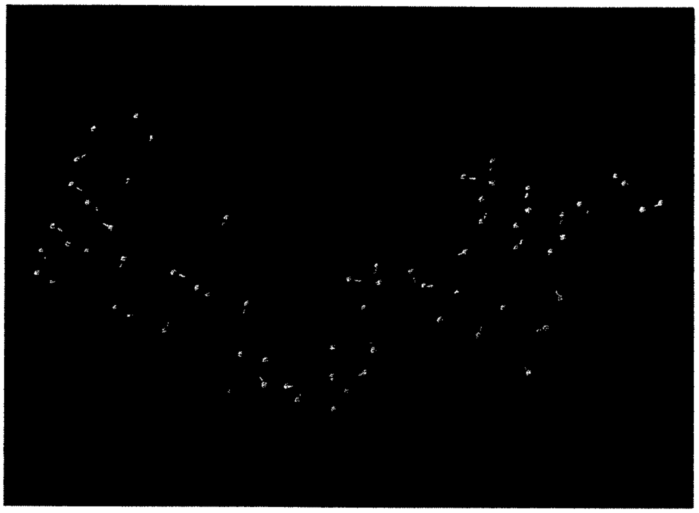

Figure 6

Molecular Structures of an RGD Containing Bioactive Polypeptide Sequence #2 (Sigma A6677)
Polypeptide Sequence: Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro (or RGD-SPASSKP)
Color Code: Grey = C, White = H, Red =O, Blue = N

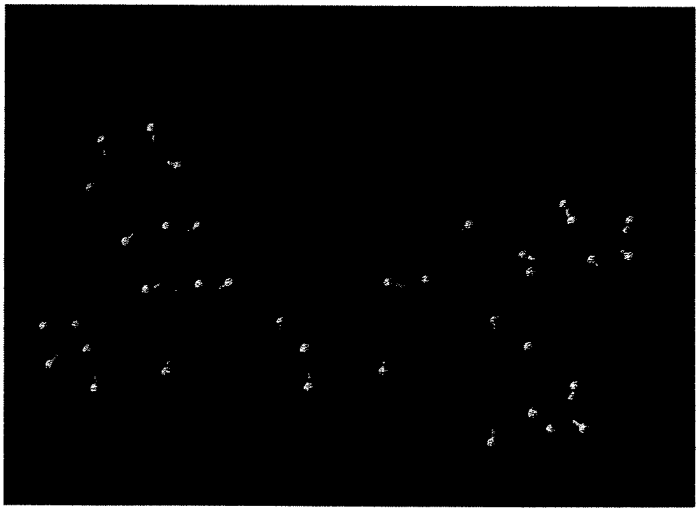

Figure 7

Molecular Structures of an RGD Containing Bioactive Polypeptide Sequence #3 (Sigma G5646)
Polypeptide Sequence: Gly-Arg-Gly-Asp-Thr-Pro (or G-RGD-TP).
Color Code: Grey = C, White = H, Red =O, Blue = N

I.3.ii.c. Covalent Coupling of Nanoparticles with Bioactive Functional Groups

The attachment of the bioactive functional compounds to the PEG moieties on the nanoparticle surface will be accomplished via a diimide catalyzed carboxylation reaction in an aqueous medium [37,38]. The reaction conditions are friendly for the adhesin compounds, so that their bioactivities should not be affected by this process. In fact, such reactions are typically used in the functionalization of peptides and natural proteins. For example, Dr. Sun's laboratory group has previously used these methods to functionalize bovine serum albumin (BSA) with derivatized fullerene molecules via diimide catalyzed carboxylation reactions [39].

I.3.ii.d. Final Nanoparticle Synthesis for Use in Subsequent Sections of the Program

Once each of the synthesis routes has been finalized for each type of functionalized nanoparticle, each type of nanoparticle will be synthesized for the in vitro bioactivity screening studies in order to identify those which most strongly bind to and agglutinate each of the targeted microbial pathogens. In addition to the functionalized nanoparticles, nonfunctionalized controls will also be synthesized for each type system which will in all ways be identical to the functional nanoparticles except that final surface groups will not be linked to the PEG side-chains. In all, approximately 24 different types of nanoparticles are planned to be initially produced: 2 cores types (polymer and oxide core) x 6 surface functionalities (sialic acid, D-mannose, 3 bioactive polypeptides, control) x 2 median sizes (250 nm and 750 nm).

The nanoparticle types exhibing the highest levels of bioactivity in the in vitro bioactivity screening tests will then be produced in large quantities for the subsequent in vivo bioactivity and exposure sentitivity studies. The details of the in vitro bioactivity screening studies are described in the following section.

I.3.iii. MATERIALS AND METHODS: PART 2. IN VITRO BIOACTIVITY STUDIES

The objective of this portion of the program is to evaluate and compare the interaction between synthesized bioactive nanoparicles and the target microorganism *Campylobacter jejuni,* in order to determine which types provide the greatest desireable bioactivity with the targeted pathogen.. The most bioactive nanoparticle types will then go on for the in vivo poultry bioactivity studies and handling sensitivity studies.

I.3.iii.a. Methods Overview

Bacterial strains selected for in-vitro study are *Campylobacter jejuni* Epi 23 [40] (poultry isolate – USDA), *Campylobacter coli* Epi 8 [40] (chicken cecal isolate – USDA), *Salmonella enteritidis* [41] (ATCC 13076), *Escherichia coli* O157:H7 [42-44] (ATCC 51658), and *Lactobacillus acidophilus* [45,46] (ATCC 53672). Viability of the tested microorganisms after interacting with the nanoparticles will be determined by direct microscope enumeration of respiring bacteria using two fluorescent dyes: 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and 4'-6 diamino-2 phenylindole (DAPI) [47-50].

I.3.iii.b. Bacterial strains and culture conditions

Campylobacter jejuni Epi 23 and *Campylobacter coli* Epi 8 will be grown in non-selective medium Brucella Broth (Difco Laboratories, Detroit, Mich.) [43] or on selective medium Pelterz's charcoal cefoperazone deoxycholate agar [47] (m-CCDA), Campy-cefex agar [47] (CCA), or charcoal-based selective medium [47] (CSM). m-CCDA is composed of campylobacter blood-free selective agar base (Oxoid Ltd., Basingstoke, UK), CCDA selective supplement (Oxoid), and yeast extract (2 g/liter) (Oxoid). CCA is composed of brucella agar (Difco Laboratories, Detroit, Mich.) (43 g/liter) supplemented with 5% laked horse blood (Oxoid), ferrous sulfate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (0.5 g/liter), sodium bisulfite (Sigma Chemical Co., St. Louis, Mo.) (0.2 g/liter), sodium pyruvate (Sigma) (0.5 g/liter), sodium cefoperazone (Sigma) (33 mg/liter), and sodium cycloheximide (Sigma)

(200 mg/liter). CSM is made of campylobacter agar base (Oxoid) and campylobacter selective supplement (Oxoid). All culturing of both *Campylobacter jejuni* and *Campylobacter coli* will be cultured at 42°C under a microaerophilic atmosphere (7% O_2, 10% CO_2, 83% N_2).

Salmonella enteritidis will be cultured in nutrient broth (Difco 0003) or on nutrient agar (Difco 0001) at 37°C. *Escherichia coli* O157:H7 will be cultured in trypticase soy broth (Difco 0370) or on trypticase soy agar (Difco 0369) at 37°C. Lactobacillus acidophilus will be cultured in lactobacilli MRS broth (Difco 0881) or on lactobacilli MRS agar (Difco 0882) at 37°C with 5% CO_2.

I.3.iii.c. Nanoparticle Slide Agglutination

Functionalized nanoparticles each containing sialic acid, D-mannose, or one of the bioactive polypeptide sequences (#1, 2, or 3) in 2 sizes (250 nm and 750 nm), will be prepared. Same-sized nonfunctionalized nanoparticles with the PEG side-chains will be used as controls in this study. Various concentrations of each test microorganism will be mixed on a microscope slide with various proportions of bioactive nanoparticle suspension and observed over oblique light (visually unaided and also under 450x, high-dry) for apparent agglutination.

I.3.iii.d. Spread plate counts

Fifty ml (10^8 CFU/ml) of an early stationary phase culture will be mixed with various compositions of synthesized nanoparticle and stirred at 50 rpm for 5 minutes at room temperature. Serial dilutions of each mixture in peptone water will be plated in duplicate on an appropriate selective agar medium. Plates will be incubated at each test microorganism's optimal condition as described above. Reduction in colony-forming units (CFU) will be recorded and compared to appropriate control (cell suspensions at the same concentrations having no nanoparticles).

I.3.iii.e. Scanning Electron Microscope Counts

Cell samples (1 ml) taken from the above mixture for scanning electron microscopy will be centrifuged for 1 min at 13,600 x g, washed in 1 ml saline, recentrifuged, and resuspended with 1-ml aliquots of 0.15 M saline. Free fluid from 0.2 to 0.4 ml of washed cell suspension will be removed by filtration through 0.8-µm-pore-size Gelman Metricel membranes; retained cells will be washed with 1 ml of saline. Washed cell samples will be fixed in 3.5% cacodylate buffered glutaraldehyde at a pH of 7.2 for 24 hours at 4°C. After removal from the fixative, samples will be dehydrated in ethyl alcohol with increasing concentrations of 50%, 70%, 85%, and 95% for 15 minutes each. Samples will then be dehydrated in 100% ethyl alcohol for two 30-minute intervals. A Jumbo Critical Point Dryer (Structure Products Inc., West Chester, PA) will be used for critical point drying of the samples. Samples will be mounted on aluminum stubs. A Hummer X sputter coater (Anatech Ltd., Alexandria, VA) will be used to coat mounted stubs with gold to an approximate thickness of 5 nm. Samples will be examined in a HITACHI 3500N scanning electron microscope (Hitachi Instruments, Inc. San Jose, CA) at an accelerating voltage of 15-25 kV and a working distance of 23-26 mm. Bacterial cell/nanoparticle aggregates will be photographically documented and compared to controls consisting only of washed cell suspensions or of nanoparticles alone.

I.3.iii.f. Double Staining (CTC-DAPI) for Viable but Non-Culturable Counts

Cell samples taken from the above *Campylobacter*-nanoparticle mixture will be stained with CTC (Polysciences, Warrington, USA) and DAPI (Polysciences, Warrington, USA) according to a modified technique of Rodriguez et al. [49]. Brain-heart infusion (500 µl, Difco) and 100 µl of a 0.05 g/L solution of pyruvic acid (Sigma) will be added to 0.5 ml of the *Campylobacter*-nanoparticle suspension in order to stimulate cellular respiration. CTC will be diluted to obtain a final concentration of 5 mM. The mixture will then be incubated for times ranging from 0.5 to 4 h at 37°C under a microaerophilic atmosphere. Cell will be subsequently harvested by filtration through an isopore polycarbonate black membrane filter (0.2 µm pore size, 25 mm diameter, Millipore) and stained with a 5 µg/ml DAPI solution for 5 min for counterstaining purposes. The stain will then be removed by filtration and the filter will be air-dried and mounted in non-fluoresent immersion oil (Olympus, Japan) before a coverslip is added. An Olympus ix70 epi-fluorescent microscope will be used to examine the filters at a magnification of 1000x. Observation will be performed with a fluorescent light fitted with a 405 nm excitation filter and a 455 nm dichroic mirror allowing simultaneous visualization of both eyes. Counting will be carried out randomly on the basis of 20 microscopic fields per filter. For each sample, four filters will be counted. Respiring cell counts showing CTC formazan crystals, and total cell counts with staining by DAPI will be determined. Viability of the cells trapped in the *Campylobacter*-nanoparticle complex will also be determined and compared to cell suspensions incubated without nanoparticles.

I.3.iv. MATERIALS & METHODS: PART 3. IN VIVO BIOACTIVITY STUDIES

The functionalized nanoparticle types exhibiting the greatest bioactivity in the in vitro cell culture studies discussed in the previous section will be then evaluated in poultry. The specific objective of this component of the overall program is to determine if bioactive nanoparticles provide a practical means of controlling *Campylobacter* colonization in poultry.

I.3.iv.a. *Chickens*

Male broiler chickens at 6 weeks of age will be housed individually in wire cages. There will be six (6) treatments as follows: 1. Control; 2. Polymer (P) gavaged at level determined by binding studies; 3. *Campylobacter* gavaged at 10^5; 4. P gavage followed by *Campylobacter* gavage; 5. P at 2X level gavage followed by *Campylobacter* gavage; 6. P at 3x level gavage followed by *Campylobacter* gavage. The experimental design will be a completely randomized design with six replicates per treatment. The initial level of gavage will be determined by results of the binding phase of the study. The feces will be collected from beneath each cage at 6, 24, 48, and 72 hours and sampled for *Campylobacter.* At the end of 72 hours each bird will be euthanized and the intestinal tract sampled for *Campylobacter.*

Fresh fecal specimens (10 grams) will be aeseptically collected for isolation of *Campylobacter jejuni.* The 10 gram sample will be inoculated into 100 ml of Brucella broth supplemented with lysed horse blood (Oxoid CM67) *Campylobacter* growth supplement (Oxoid SR117) and *Campylobacter* slective supplement (SR117). Incubation of the enrichment broth will be performed under a microaerophilic environment at 37 C for 4 hours, then continued at 42 C for 20 hours. Serial dilutions of the enrichment broth will be made and 100 *ul* of each dilution will be plated on *Campylobacter* CVA agar or Campy Cefes agar. All plates will be incubated at 42 C for 48 hours in a microaerophilic environment provided through the use of *Campylobacter* microaerophilic gas generating systems in environmental jars. Suspect *Campylobacter* colonies will be confirmed with gram-staining and the *C. jejuni* latex agglutination test.

A second chicken trial will be conducted with broiler chickens reared from hatch until 7 weeks of age (market age). The birds will be reared in a curtain sided facility similar to that used by the chicken broiler industry. There will be 36 pens with pine shavings used as bedding material (litter). All pens will have litter used for a previous flock. There will be six (6) treatments as follows: 1. Control; 2. Polymer fed to chickens in feed from day of hatch until 7 weeks of age; 3. Polymer at 2x level fed to chickens in feed from day of hatch until 7 weeks of age; 4. Polymer at 3x level fed to chickens in feed from day of hatch until 7 weeks of age; 5. Polymer at 2x level fed to chickens in feed from 6 to 7 weeks of age; and 6. Polymer at 3x level fed to chickens in feed from 6 to 7 weeks of age. The experimental design will be a randomized block design with six replicate pens per treatment. The birds will be fed a typical broiler industry series of starter, grower, and finisher feeds. All birds will be weighed by pen at placement and at 3 and 7 weeks of age. Feed consumption will be determined by pen at 3 and 7 weeks of age. Mean bird weight and feed conversions (period and cumulative) will be determined. Three birds per pen will be euthanized at 7 weeks of age and the intestinal tract will be sampled for *Campylobacter* organisms using standard recovery procedures as described above.

I.3.iv.B. *Turkeys*

Male turkey poults at 4 weeks of age will be housed individually in wire cages. There will be six treatments as follows: 1. Control; 2. Polymer (P) gavaged at level determined by binding studies; 3. *Campylobacter* gavaged at 10^5; 4. P gavage followed by *Campylobacter* gavage; 5. P at 2X level gavage followed by *Campylobacter* gavage; 6. P at 3x level gavage followed by *Campylobacter* gavage. The experimental design will be a completely randomized design with six replicates per treatment. The feces will be collected from beneath each cage at 6, 24, 48, and 72 hours and sampled for *Campylobacter.* At the end of 72 hours each bird will be euthanized and the intestinal tract sampled for *Campylobacter* organisms using standard recovery procedures as described above.

I.3.v. MATERIALS & METHODS: PART 4. HUMAN HEALTH AND SAFETY STUDIES

The objectives of this portion of the program are to provide initial screenings studies (both in vitro and in vivo) related to the health and safety of human exposure to the synthesized bioactive nanoparticles.

I.3.v.a. Cell Culture Testing (MTT) of Functionalized Nanoparticles

Cell toxicity will be conducted for each type of functionalized nanoparticle selected from the results of the in vitro bioactivity studies (Section I.3.iii) plus the nonfunctionalized controls by direct human cell/nanoparticle contact experiments using the MTT test method [51,52]. Cell toxicity experiments will be conducted by dissolving 5 mg of sterile nanoparticles (ethylene oxide sterilized) in 5 ml of sterile physiologic saline under sterile conditions and stirred to produce an aqueous nanoparticle suspension. Cell toxicity will be performed using representative human cell lines: human lung fibroblasts (ATCC CCL-171), human dermal fibroblasts, human corneal epithelial cells (ATCC CRL-11515), and human colon fibroblasts (ATCC CRL-1459). The MTT-test method will be applied by exposing the cells to the nanoparticle containing solution over a series of dilutions. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid) is a tetrazolium salt. Cells are able to reduce this salt in their mitrochondria to a formazan precipitate by way of membrane bound NADPH+H^+ oxidases, which are also involved in glucose-6-phosphaste oxidation. The formazan can be solved with 2-propanol and be quantified spectrophotometrically (λ = 590 nm). The reduction in absorbance is a measure for the biochemical activity of a cell and will be recorded along with visual observation of cell proliferation response to exposure to the test materials. Positive controls (Thermanox® polystyrene) and negative controls (copper) will be used for relative comparison with cellular responses to the nanoparticles.

I.3.v.b. In Vivo Acute Exposure Sensitivity Testing of Functionalized Nanoparticles

Overview: An evaluation of acute toxicity data will evaluate the relationships, if any, between the exposure of animals to the test substances and the incidence and severity of any abnormalities, including behavioral and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects.

In the acute sensitivity test, the most promising bioactive nanoparticles as detemined by the in vitro bioactivity assays (Section I.3.iii) and nonfunctionalized nanoparticle controls will be used in the acute exposure sensitivity studies. Four types of tests will be conducted to acutely assess oral, inhalation, skin, and ocular sensitivity to nanoparticle exposure. Healthy young adult rats (8-12 weeks of age, within 20% of mean weight, two male and two female) will be used as the test animal for both the oral and inhalation sensitivity tests, while healthy young adult rabbits (at least 12 week of age within 20% of mean weight, two male, two female) will be used for the skin and ocular sensitivity tests. Each type of nanoparticle (assuming 3 or 4 types selected from Section I.3.iii plus 1 or 2 controls) will be administered in two dose levels applied to four animals each, for a total of approximately 40 animals total per exposure test type (i.e. 40 rats each for oral and inhalation studies; and 40 rabbits each for the skin and ocular studies). A group of 4 animals for each test type will also be studied which do not receive any nanoparticles as an additional control group. Experimental room temperature will be maintained at 22 +/- 3 °C with the relative humidity kept between 30-70% and a 12/12 light/dark cycle will be used. Conventional laboratory diets are used with an unlimited supply of drinking water. Animals will be housed in cages in minimal numbers such that each individual animal can be clearly observed. Animals will be observed at least twice daily and their behavior recorded during and after the testing periods until euthanization. All procedues will, of course, be cleared through the appropriate internal review panels prior to commencing these studies.

Acute Oral Sensitivity Tests [51,53] Four rats in each test group will be dosed by the adminstration of oral gavage in two dose levels (500 and 1000 mg/kg as an aqueous slurry at 250 and 500 mg/ml nanoparticle concentration), with one dose being applied per group per day for three consecutive days. The animals will be observed for 14 days after treatment and then euthanized for necropsy.

and Animal Facilities Management on a regular basis to assure that procedural compliance is maintained throughout the studies.

I.3.vi. Problems That Might be Encountered in Research Program

In a novel approach such as the one proposed, it is difficult to foresee all of the problems that might arise. In Part 1 of the proposed research, the synthesis and photocuring of the nanoparticles into size ranges and morphology, is a relatively straightforward process. There is a wide selection of polymers which are appropriate candidates for the core structure. The para-functionalized polystyrene polymers have been chosen as initial candidates for nanoparticle constructure for their low cost and structural simplicity.

In Part 2, the data from the spread plate counts of the nanoparticle slide agglutinations will be difficult to interpret as to whether the enteropathogens have actually been reduced in viable count or are merely agglutinated, yet still viable. It is quite possible that the bacterial cells may continue to divide while agglutinated with the nanoparticles, since the nanoparticles have no obvious toxic properties. Obviously, a mass of clumped viable cells will form a single colony indistinguishable for a colony arising from a single viable cell. The use of the double staining method with the tetrazolium chloride and the 2 phenylindole will be used to microscopically differentiate between viable and dead cells. While the object of the exercise is to select those nanoparticles which most avidly clump the bacterial cells, their very success in this regard may complicate the accurate quantitation of the ratio of dead to viable cells. It might very well be difficult to ascertain viability status microscopically if the nanoparticles cause large, three-dimensional clumps of bacteria.

I.3.vii. Possible Limitations to the Proposed Approaches

The greatest anticipated limitation on the efficacy of the proposed nanoparticle strategy is the microbial complexity of the avian intestinal tract. The microflora in the digestive system of chickens is remarkably diverse. The cecum is a region which provides a stable microbial habitat, and contains a dense population of obligate anaerobes ranging from 10^9 to 10^{11}/g cecal contents [60]. There is a distinct probability that the nanoparticles, despite their specificity for campylobacter adhesins, will also agglutinate bacteria other than campylobacters (or other targeted enteropathogens). The two major factors affecting cecal microflora are diet (including antibiotics) and age of the chicken. For the nanoparticle strategy to be consistently effective in purging the targeted pathogen, it may be necessary to withhold prior to nanoparticle administration. It may also be necessary to limit treatment to a narrow chronological "window of opportunity", i.e., a certain age of bird. These factors will be assessed during the in vivo bioactivity studies of this proposed overall program.

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1.4. CURRENT WORK

Dr. Stutzenberger, Professor of Microbiology and Molecular Medicine at Clemson University, has gained extensive research experience in working with enteropathogens since 1994. He was co-principle investigator on a contract with Oravax, Inc to develop an oral vaccine for *Helicobacter pylori*, a campylobacter-like bacterium causing active gastritis and duodenal ulcers in humans. Dr. Stutzenberger and colleagues at Clemson University developed the first small primate animal (squirrel monkey) model to study the *H. pylori* infection. His later training in working with *H. pylori* at the Women's and Children's Hospital in Adelaide, Australia extended his experience in research on human enteropathogens in small animal models. Recently, his enteropathogen research has been expanded to include *Aeromonas caviae* and *A. hydrophila* (both causative agents of diarrhea in humans and animals) and the vectors by which these bacteria are spread in the environment. The experience that has been gained in the culturing and manipulation of these enteropathogens will be valuable in the successful completion of the proposed research.

Dr. Latour, Associate Professor of Bioengineering and Materials Science & Engineering at Clemson University, currently directs an active research program focusing on biomolecular-surface interactions. He is specifically involved in quantitatively studying submolecular events which control protein-surface adsorption and ligand-receptor binding, and the implementation of this understanding toward the design of biactive surfaces for the control of cellular responses. Applications of this research include the design of medical implant surfaces for enhanced biocompatibility, the design of tissue engineering substrates for cellular control, and design of bioactive nanoparticles for drug delivery, biological weapons defense, and food safety. Dr. Latour's expertise in the thermodynamics of intermolecular interactions and bioactive surface design provide an essential component of the proposed program for the design of bioactive nanoparticles for the binding of targeted microbial pathogens.

Dr. Sun, Professor of Chemistry at Clemson University, presently conducts an extremely active research program at Clemson University which focuses on the development of fabrication methods for the production of nanostructures, including nanoparticles, nanotubes, and nanocomposites. Dr. Sun's research group has developed several novel techniques for the synthesis of nanoparticles from metal oxides, polymers, and carbon ceramics. Dr. Sun is also very involved in nanoparticle functionalization research which involves the linking of desired chemical functional groups and molecules to nanoparticle surfaces to achieve desired nanoparticle activity. His broad expertise in the fabrication of nanoparticles provides an essential component for the development of the spectrum of nanoparticles proposed in this research program.

Dr. Tzeng, Instructor of Microbiol and Molecular Medicine at Clemson University, has recently completed his graduate work in microbiology and is currently serving as a research and teaching associate in Microbiology. Dr. Tzeng has a great deal of expertise in working with bacterial cell culture systems and his recent work has involved the isolation, identification, and maintenance of aerobic, microaerophilic, and anaerobic bacteria, and the use of various bacterial transformation techniques (electroporation, conjugation, viral transformation) and immunological techniques (Western, ELISA, mono- and polyclonal antibodies). In addition, Dr. Tzeng has several years practical experience with scanning electron microscopy, transmission electron microscopy, and fluorescence microscopy techniques for the assessment of bacterial systems.

Dr. Grimes, Associate Professor of Poultry Science at NC State University, is currently involved in several aspects of poultry research directed toward increasing health and production. Specific studies involve the effects of food supplements on growth and reproductive capabilities. He is also involved in the development of alternative strategies to the use of antibiotic treatments to maintain health and improve growth characteristics in poultry. His expertise in the testing and evaluation of poultry will provide an essential component of this overall program which directly links the developed technology to its field use and extension service.

Dr. Rodgers is currently the Director of the Clemson Institute of Environmental Toxicology and the Chair of the Department of Environmental Toxicology at Clemson University. Dr. Rodgers' research focus lies in the area of the evaluation of herbicides and pesticides on aquatic animal life. Although not directly related to human health and safety, the Institute of Environmental Toxicology is very experienced in conducting health and exposure assessment studies using rat and rabbit models. The Institute maintains a full-time staff to assist with animal model studies including quality assurance/quality control personnel to ensure full compliance in all animal related procedures. These resources will serve to ensure proper protocols are followed for the in vivo exposure sensitivity studies.

As indicated in the preceding paragraphs, the research team assembled for the proposed program maintains a high-level of professional experience and expertise in areas closely related to the proposed studies and is fully capable of efficiently and effectively carrying out the planned research program. The interactions between each of the members in this proposed program are further described in the following section.

1.5. COOPERATION AND INSTITUTIONAL UNITS INVOLVED

Rationale for Collaboration between Clemson and NCSU

Clemson University serves as the land grant institution of South Carolina and consequently has a large agricultural component in its mission of education, research and extension service to the public. Therefore, the proposed research is highly appropriate to Clemson's land grant tradition. The Department of Microbiology and Molecular Medicine is positioned in the College of Agriculture, Forestry and Life Sciences and has the only complete microbiology program (BS, MS, PhD) in the state. Dr. Stutzenberger (Co-P.I.) has had experience in working with an enteric pathogen (*Helicobacter pylori*) in animal models (mice and squirrel monkeys); nine publications in international refereed journals have been generated from his studies in the last five years. Recently, his research interest has broadened to include *Aeromonas spp.* and the epidemiological role of houseflies as a vector in their dissemination. This experience will be a valuable asset to the program and will be additionally complemented by the efforts of Dr. Tzeng, also of the Department of Microbiology.

Dr. Stutzenberger's expertise in working with enteropathogens in animal models will be complemented by collaboration with Dr. Robert Latour (Dept. Bioengineering) and Dr. Ya-Ping Sun (Dept. Chemistry) in the College of Engineering and Sciences, both of which direct their own very active research programs. Dr. Sun's expertise lies in the design and synthesis of functionalized nanoparticle systems. In this multidisciplinary program, Dr. Sun's experience will be combined with Dr. Latour's expertise in submolecular-level thermodynamics and biomolecule-surface interactions for the design of novel synthetic bioactive nanoparticles targeting *Campylobacter jejuni.*

Dr. Jesse Grimes and colleagues (Dr. Bryan Sheldon and Dr. Mike Wineland) at North Carolina State University provide the practical experience and facilities for working with large numbers of experimental poultry during the *in vivo* phase of nanoparticle testing. In particular, Dr. Grimes' experience with dietary effects in both chickens and turkeys will be a valuable asset in defining any overt nutritional effects which nanoparticles might have in these birds. Moreover, Dr. Grimes' extension position in the NCSU Dept. Poultry Science will facilitate an integrated multifunctional research/extension approach to the proposed research.

The final component of this overall program involves the assessment of the handling and exposure sensitivity of the designed nanoparticle systems. Although the nanoparticles will be chemically inert and will not contain toxic chemicals, this is a very important component of this overall program in order to assess and confirm the safety of freely handling and openly using the designed bioactive nanoparticles. This component of the program will be overseen by Dr. Latour of the Department of Bioengineering and Dr. Rodgers of the Department of Environmental Toxicology with the animal studies being conducted in the centralized Godley-Snell Research Center at Clemson University.

1.6. EQUIPMENT AND FACILITIES:

The proposed will be undertaken at Clemson University in both the College of Engineering and Sciences (incorporating the Departments of Bioengineering and Chemistry) and the College of Forestry, Agriculture, and Life Sciences (incorporating the Department of Microbiology and Molecular Medicine). General support facilities within both of these colleges will be provided to support the proposed program. In addition, extension work will be conducted through Prof. Jesse Grimes, NC State University.

The two participating colleges maintain a very broad range of facilities in support of education and research which are too numerous to list within space limitation of this proposal. Facilities include s**pectroscopy instruments:** e.g. UV-VIS, IR, FT-NMR, Phosphorescence/Fluorescence, NMR, FTIR: ATR & DRIFTS; **Thermal Analysis Instruments:** DTA, DSC, DMA; **Separation Science and Molecular Identification:** Capillary GC with Head Space, HPLC/GPC, Dynamic Contact Angle Analyzer, GC-MS, UV-VIS spectrophotometers; and **Microscopy Instruments**: Auger Microprobe with SIMS and Backscatter, High-Precision SEM with EDX, ESCA, and confocal microscope (anticipated new purchase). Life Sciences also maintain a wide-range of support facilities for biomolecular and cellular studies.

Some of the specific items of equipment and facilities are listed for each section of the proposed program below.

I. Nanoparticle Synthesis:

I.i. Equipment for Supercritical Fluids and Photochemical Reactions

RESS/RESOLV apparatuses for the preparation of nanoparticles and nanocomposites.

Variable-volume apparatuses for measuring phase behavior/solubilities in supercritical fluids.

High-pressure apparatuses for spectroscopic studies of supercritical fluids at ambient and high (up to 500 °C) temperatures.

Photochemical setup for conventional photochemistry and for photochemical reactions under high-pressure/high-temperature conditions; optical cells designed for high-pressure/high-temperature photochemical reactions; optical fibers-coupled reactors for high-pressure/high-temperature photochemistry.

I.ii. Facilities for Testing and Analysis

***Optical Spectroscopy*:**

Shimadzu UV-3100 UV/Vis/NIR spectrophotometer with the attachment for solid samples; Shimadzu UV-2101PC UV/Vis spectrophotometer.

Spex Fluorolog-2 emission spectrometer equipped with two detectors for regular and near-IR sensitive (thermoelectrically cooled, up to 1,200 nm) measurements.

Nanosecond time-resolved emission spectrometer with thermoelectrically cooled detectors.

Detectors, electronics, and optics for nanosecond and picosecond optical limiting and Z-scan experiments.

Setups for nanosecond and picosecond transient absorption measurements.

Apparatus for picosecond fluorescence decay studies.

Nicolet FT-IR instrument.

Spex Raman spectrometer with 1 meter monochromator and Princeton Instruments CCD detector.

Thermal Analysis:
TA Instruments for thermal gravimetric analysis and differential scanning calorimetry.

Separation and Identification:
Two medium-pressure liquid chromatography systems.
HPLC system for analytical analysis and GPC polymer characterization.
Preparative HPLC system (100 mL/min capability) with preparative and specialty columns.
Fisons medium resolution GC-MS (This machine is old, with a lot of problems. Because the vendor has decided not to support this product, the maintenance for normal operation has become very difficult. Thus, the acquisition of a new GC-MS system is requested in the budget for the proposed project).

Other Specialty Equipment:
Apparatus for photoconductivity measurements.
Kratos MALDI time-of-flight MS.
Quantum Design SQUID MPMS-5S magnetometer.

Laser Equipment:
Coherent Antares mode-locked Nd:YAG laser (76 MHZ, 80 ps).
Two Coherent 700 series ultra fast dye lasers.
Continuum RGA 60 series regenerative amplifier.
Coherent Innova 70 Argon-Ion laser system.
Continuum Surelite-I pulsed Nd:YAG laser (10 Hz, 5 ns).
Hamamatsu solid-state laser (picosecond light pulser).
Several optical tables.

NMR Facility:
Bruker 200-MHZ multinuclear NMR spectrometer with solid state capabilities.
Bruker 300-MHZ multinuclear NMR spectrometer.
JEOL 500-MHZ multinuclear NMR spectrometer.

X-ray Facility:
Three single crystal instruments; Rigaku AFC-7R(18kW), Siemans R3mV with liquid nitrogen based low-temperature systems, and a Syntex P2(1).
Powder X-ray diffraction Scintag XDS 2000 with variable temperature stage and thin-film attachment.
CCD-area detector based single crystal system with liquid helium cooling.

Microscopy Facility:
JEOL 848 high-precision scanning electron microscope with energy dispersive X-ray analyzer.
Hitachi 600 AB 100 kV transmission electron microscope with energy dispersive X-ray analyzer.
Hitachi 7000 125 kV transmission electron microscope.

II. In Vitro Bioactivity Studies

Facilities available in the Department of Microbiology and Molecular Medicine, Clemson University, College of Agriculture, Forestry, and Life Sciences:

II.i. Laboratory:

The research space allotted to the Co-P.I. is divided into two lab areas totaling about 142 meters^2, located in 21-year old Jordan Hall. Our department has all the general facilities required for microbiological and chemical studies. The university electron microscope facility is also located in Jordan Hall.

II.ii. Computer:

All office and lab computers (total of three in our lab) are all connected to the university main frame system through which access to on-line literature searches, e-mail, statistical analysis and internet services are provided.

II.iii. Office:

Our departmental offices are equipped with all necessary communication equipment to provide support services for this project.

II.iv. Other:

Our college machine shop and electronics shop has three and four experienced technicians, respectively.

II.v. Major equipment:

There is free access to low speed and preparative ultracentrifuges, spectrophotometers, electrophoresis units (analytical and preparative), sonic disintegrators, gel scanners and Kodak imagers, incubators, laminar flow hoods, shaking water baths, pH meters, and two thermcyclers. All of this equipment is contained either in Dr. Stutzenberger's laboratories, in the equipment room adjacent to his lab rooms or in the departmental media preparation complex on the same hall.

III. In Vivo Bioactivity Studies in Poultry at NC State (subcontract)

NCSU has all bird rearing and handling facilities located either in Scott Hall or at the NCSU Chicken Educational Unit or at the NCSU Turkey Educational Unit.

NCSU also has fully appropriate laboratories and equipment for harvesting and enumerating microbes and for any other associated lab work proposed in this program.

Further detailed equipment lists are available if specifically requested.

IV. In Vitro Cell Toxicity Studies

IV.i. Cell Culture Laboratory

- Autodessicators (Scienceware Bel-art Products)
- Autoclave (Market Forge Industries, Inc.)
- Nano Pure water purifiers (1 Barnstead, 1 Millipore)
- Biohazard hoods 1- 4ft A/B-3; 1-4ft A/B
- Chemical fume hoods, 8 ft
- Analytical balances (Denver Instr., Mettler Instr.)
- Incubators, tissue culture (NuAire, VWR)
- Centrifuge, refigerated bench model, swing bucket (International Equipment Co)
- Microscope, inverted, epi-fluorescent with CCD camera (Nikon)
- Image Pro Plus Software, Image Analysis for Microscope (Media Cybernetics)
- Microscope, cell culture examination (Nikon)
- Photomicrographic camera (Nikon)
- UV-VIS Spectrophotemeter with analysis software (Beckman)
- pH meters

IV.ii. Histopathology Laboratory

- Complete EXAKT® System for hard tissue histology
- Assortment of Grinding and Polishing Equipment
- Polycut E sliding microtome
- BUEHLER Isomet 2000
- Ziss micron cryostat
- TISSUE-TEK® VIP Tissue Processor
- Polycut E sliding microtome

In Vivo Contact Sensitivity Studies

Animal studies will be conducted in the Godley-Snell Research Center at Clemson University. The Godley-Snell Research Center (GSRC) opened on Clemson University campus in August of 1995 as a centralized animal research facility. GSRC has 22,000 sq. ft. dedicated to maintaining a variety of laboratory animal species used in biomedical research programs at Clemson University; and to providing resources and support facilities for those programs. GSRC is managed by the Department of Research Services which provides facilities, equipment, caging, and veterinary and technical support to facilitate animal research programs.

GSRC has housing space for large and small laboratory animals, including two suites of individually ventilated isolation cubicles. Facilities and caging are available for conventional laboratory housing for rodents, rabbits, chickens, goats, swine and other species as required.

Laboratory animals are procured from pre-approved sources of specific pathogen free animals. Research Services staff includes laboratory animal trained veterinarians and animal care technicians. Animal care staff provide seven day a week animal care and health survelliance.

GSRC maintains a biocontainment suite that meets CDC, Animal Biosafety Level 3 criteria.

Access to the facility is controlled by an electronic card access system. The suite contains (1000 sq. ft) animal housing space, a controlled environment chamber, laboratory space and personnel shower/locker. Laboratory equipment includes a Class II B2 biosafety cabinet and a pass-through steam sterilizer. The biocontainment area is maintained under negative pressure differential with 100% fresh air and 100% HEPA filtered exhaust to outside.

GSRC maintians a fully equipped central surgical facility with two double operating rooms, nursing station, sterile prep, recovery room, procedure and surgical prep room, and radiology. Anesthetic delivery and monitoring equipment are available for various animal species.

Animals are maintained in accordance with all animal welfare regulations and federal guidelines to ensure humane care. All of Clemson University animal research facilities and programs have received full accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

The Department of Research Services strives to provide and promote the highest standards of animal care and the humane and responsible use of animals in the advancement of knowledge which will benefit the health or welfare of man or animal.

1.7. PROJECT TIMETABLE

	Year 1				Year 2				Year 3			
TASK	1	2	3	4	1	2	3	4	1	2	3	4
I.A. Bioactive Nanoparticle Synthesis	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx		
I.B. In Vitro Bioactivity Assays			xxx	xxx	xxx	xxx	xxx	xxx	xxx			
I.C. In Vivo BioactivityAssays					xxx	xxx	xxx	xxx	xxx	xxx	xxx	
I.D. In Vitro Exposure Sensitivity Assays.					xxx	xxx	xxx	xxx	xxx			
I.E. In Vivo Exposure Sensitivity Assays									xxx	xxx	xxx	
Final Report												xx

II. APPENDICES

Copies of the following two reprints of publications are provided in this section.

II.1. Nanostructure Synthesis Paper

Sun Y.-P., Lawson G.E., Huang W., Wright A.D., and D.K. Moton, Preparation and Characterization of Highly Water-Soluble Pendant Fullerene Polymers, Macromolecules, 32: 8747-8752 (1999).

II.2. Bacterial Adhesion Paper

Boland T, Latour RA, and Stutzenberger FJ: Molecular Basis of Bacterial Adhesion, in *Handbook of Bacterial Adhesion - Principles, Methods, and Applications,* An YH and Friedman RJ (eds), Chapter 2, Humana Press, Totowa, NJ, 2000, pp: 29-41.

Macromolecules **1999**, *32*, 8747

Preparation and Characterization of Highly Water-Soluble Pendant Fullerene Polymers

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ABSTRACT: Pendant fullerene polymers were prepared by attaching methano[60]fullerene carboxylic acid (MFCA) and methano[60]fullerene phenylpropyl carboxylic acid (MFPCA) to linear poly(propionylethylenimine-*co*-ethylenimine) (PPEI–EI) via amide linkages in carbodiimide-catalyzed coupling reactions. The pendant MFCA–PPEI and MFPCA-PPEI polymers, which were characterized using NMR, gel permeation chromatography, FT-IR, and other instrumental methods, most likely have a "charm bracelet" type structure without significant cross-linking. MFCA and MFPCA were also coupled with diethylamine in the same carbodiimide-catalyzed reactions, and the methano[60]fullerene amides thus obtained were used as monomeric model compounds for the pendant polymers. UV–vis absorption spectra of the polymer-bound fullerenes, though broader, retain the characteristic features observed in monomeric methano[60]fullerene derivatives. The pendant MFCA–PPEI and MFPCA–PPEI are highly water-soluble, with the equivalent aqueous solubilities of the polymer-bound fullerene cages significantly higher than that of the parent [60]fullerene in room-temperature toluene. Thus, the preparation of pendant fullerene polymers represents an effective approach for introducing fullerene cages into an aqueous solution or hydrophilic environment.

Introduction

Fullerene cages are highly hydrophobic, insoluble in water and most polar solvents. Several approaches have been explored for the introduction of fullerenes into an aqueous or polar environment.[1] For example, water-soluble fullerene carboxylic acid derivatives,[2–5] fullerenols,[6] and fullerene amino acid derivatives[7,8] have been prepared and used in the investigation of biochemical and medicinal activities of fullerene cages. Peptide functionalized fullerene derivatives and fullerene–oligonucleotide conjugates have also been studied for their biologically significant functions.[9–11] In addition, the preparation and properties of fullerene–protein conjugates with high fullerene/protein molar substitution ratio have been reported.[12]

The incorporation of fullerene cages into polymeric structures is another approach for aqueous solubilities.[13–16] For example, polyethers containing amino end groups were used to functionalize fullerene cages.[13] The polymeric fullerene materials thus obtained are amphiphilic, but the polymer structures are complex due to extensive cross-links.[13] To improve the selectivity in the attachment of fullerene cages to polymer, "bucky-ball-fishing" approaches on the basis of photochemical amination[14] and catalytic amidation[15] reactions were used to prepare pendant fullerene–aminopolymers without significant cross-linking. The pendant fullerene–aminopolymers thus obtained were highly water-soluble.[14,15] In the absence of cross-linking, pendant fullerene polymers are dubbed as "charm bracelet" fullerene polymers.[14,15,17–24] Generally speaking, as a result of relatively simple fullerene–polymer linkages in pendant fullerene polymers, the fullerene cages are largely unaffected following the attachment to polymer. Thus, the preparation of pendant fullerene polymers represents an effective method for introducing fullerene cages into polymeric systems in a structurally less invasive and more controllable fashion.

Here we report the preparation and characterization of highly water-soluble pendant fullerene polymers with well-defined structures. The polymers were obtained from carbodiimide-catalyzed amidation reactions of methano[60]fullerene carboxylic acid (MFCA) and methano[60]fullerene phenylpropyl carboxylic acid (MFPCA) with poly(propionylethylenimine-*co*-ethylenimine) (PPEI–EI), followed by the amidation of unused secondary amine units on the polymer backbone with propanoic acid. The specificity of the amidation reactions with respect to the fullerene–polymer linkages results in "charm bracelet" type pendant polymer structures. Also reported are results from the characterization of the pendant MFCA–PPEI and MFPCA–PPEI using ^{1}H and ^{13}C NMR, gel permeation chromatography (GPC), FT-IR, and other instrumental techniques. The polymer structures and properties are compared with those of the monomeric model compounds prepared in the same carbodiimide-catalyzed amidation reactions of MFCA and MFPCA with diethylamine. Aqueous solubilities of the pendant fullerene polymers and related issues are discussed.

Experimental Section

Materials. Fullerene C_{60} (99.5%) was obtained from the Southern Chemical Group and BuckyUSA. Toluene was distilled after being refluxed with sodium wire. CS_2 was fractionally distilled before use. All other chemicals and solvents were used either as received or after simple purification. Deuterated solvents and tetramethylsilane (TMS) for NMR experiments were obtained from Cambridge Isotope Laboratories. Polystyrene standards with molecular weights M_W of 3900–965 600 and polydispersity of 1.15 and less and poly(2-vinylpyridine) standards with molecular weights M_W of 9100, 50 000, and 243 000 and polydispersity of 1.18 and less were purchased from Polymer Source Inc.

Poly(propionylethylenimine) (PPEI) with an average molecular weight M_W of ~50 000 was obtained from Aldrich and purified before use. In the purification, a PPEI sample was dissolved in acetonitrile to form a concentrated solution and

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then precipitated into diethyl ether. After repeated precipitations, the polymer sample was dried in a vacuum oven. ^{1}H NMR (300 MHz, $\mathrm{CDCl_3}$): δ 3.46 (bs, 2 × $\mathrm{NCH_2}$), 2.35 (bs, $\mathrm{COCH_2CH_3}$), 1.12 (bs, $\mathrm{COCH_2CH_3}$) ppm. ^{13}C NMR (75 MHz, $\mathrm{CDCl_3}$): δ 174 (C=O), 44 (2 × $\mathrm{NCH_2}$), 26 ($\mathrm{COCH_2CH_3}$), 9.3 ($\mathrm{COCH_2CH_3}$) ppm.

Measurements. Two GPC setups were used in the polymer analyses. One of them using chloroform as mobile phase consists of a Waters 510 analytical HPLC pump and Waters UV–vis and refractive index detectors. GPC separations were achieved by use of three Waters HT-6E linear Styragel columns connected in a serial fashion, with chloroform flow rate of 1 mL/min. Linear polystyrene standards were used as molecular weight references. The other GPC setup uses DMSO as mobile phase. It consists of a Shimadzu LC-10AS analytical HPLC pump and a Rainin Instrument UV-1 UV–vis detector. Two serially connected Waters HT-6E linear Styragel columns prepared in DMF were converted to DMSO by pumping a low volume of DMSO (0.1 mL/min) for 24 h at 75 ℃. The temperature of the DMSO column and a precolumn loop was maintained by use of a tube-oven made in house. The mobile phase DMSO containing 0.2% lithium bromide salt was pumped at 1 mL/min. Linear poly(2-vinylpyridine) standards were used as molecular weight references.

^{1}H and ^{13}C NMR spectra were obtained on a Bruker AC-300 MHz NMR spectrometer and a JEOL Eclipse +500 NMR spectrometer. Tetramethylsilane was used as an internal standard.

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS analyses were performed on a Kratos Kompact-III mass spectrometer equipped with a nitrogen laser. Each sample was measured in positive ion mode and/or negative ion mode in reference to $\mathrm{C_{60}}$ as an internal standard. Several compounds including α-cyano-4-hydroxycinnamic acid, 3,5-dimethoxy-4-hydroxycinnamic acid, 2,5-dihydroxybenzoic acid, pyrene, and 9-nitroanthracene were tested as matrix materials, and pyrene was found to be the best. Thus, the results reported in this paper were obtained using pyrene as the matrix.

Absorption spectra were obtained on Shimadzu UV-2101PC and UV-3100 spectrophotometers. FT-IR spectra were measured on a Nicolet Magna-IR 550 FT-IR spectrometer. A KBr plate with the sample deposited on the surface or a KBr pellet homogeneously dispersed with the sample were used in the measurements.

Poly(propionylethylenimine-*co*-ethylenimine) (PPEI-EI). PPEI-EI random copolymer was prepared via a partial hydrolysis of PPEI under acidic conditions. In a typical reaction, 1 g of PPEI was dissolved in 50 mL of hot water. To the solution was added 2 mL of 1 M HCl solution. After refluxed with stirring for 10 h, the aqueous solution was neutralized by adding NaOH solution to adjust pH to ~10. Upon the removal of water, PPEI-EI was extracted into chloroform. Further purification was accomplished by precipitating the polymer from a concentrated chloroform solution into hexane. After drying under vacuum at 50 ℃, a glassy PPEI-EI sample was obtained. ^{1}H NMR (300 MHz, $\mathrm{CDCl_3}$): δ 3.45 (bs, 2 × $\mathrm{NCH_2}$), 2.78 (bs, 2 × $\mathrm{NHCH_2}$), 2.4 (bs, $\mathrm{COCH_2CH_3}$), 1.12 (bs, $\mathrm{COCH_2CH_3}$) ppm. ^{13}C NMR (75 MHz, $\mathrm{CDCl_3}$): δ 174 (C=O), 48.5 (2 × $\mathrm{NHCH_2}$), 44 (2x$\mathrm{NCH_2}$), 26 ($\mathrm{COCH_2CH_3}$), 9.3 ($\mathrm{COCH_2CH_3}$) ppm.

The degree of hydrolysis x_{NH} (mole fraction of $-\mathrm{CH_2NHCH_2}-$ units in the copolymer) was controlled via varying the reaction time. The x_{NH} values were estimated using ^{1}H NMR signal integrations.[14] Since the signals at 3.45, 2.78, and 1.12 ppm are due to $\mathrm{CH_2NCOC_2H_5CH_2}$, $\mathrm{CH_2NHCH_2}$, and $\mathrm{NCOCH_2CH_3}$ protons, respectively, the ratio between numbers of NH and $\mathrm{NCOCH_2CH_3}$ units r_0 can be estimated as follows.

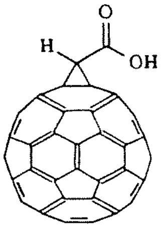
$$r_0 = I_{2.78}/I_{3.45} \tag{1a}$$

or

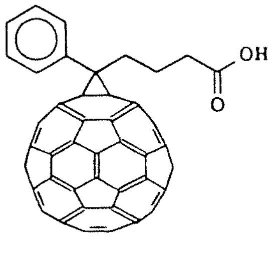
$$r_0 = {}^{3}/_{4}(I_{2.78}/I_{1.12}) \tag{1b}$$

where I represents the proton signal integrations. Thus, $x_{\mathrm{NH}} = r_0/(1 + r_0) \times 100\%$. The results from eqs 1a and 1b agree well. PPEI–EI copolymer samples with x_{NH} of 3%, 7%, and 14% were obtained.

Methano[60]fullerene Carboxylic Acid (MFCA) and Methano[60]fullerene Phenylpropyl Carboxylic Acid (MFPCA). MFCA and MFPCA were synthesized using procedures similar to those reported in the literature.[25-28]



MFCA



MFPCA

Pendant MFCA–PPEI. MFCA was coupled with PPEI–EI to form pendant MFCA–PPEI–EI using 1-ethyl-3-(dimethylaminopropyl)carbodiimide (EDAC) as a coupling reagent.[15,29] In a typical reaction, EDAC (24 mg, 0.13 mmol) was added to a solution of MFCA (25 mg, 0.032 mmol), PPEI–EI (100 mg, x_{NH} = 3%), and triethylamine (16 μL, 0.12 mmol) in 12 mL of a bromobenzene–chloroform (3:1 v/v) mixture. The solution was stirred at 0 ℃ for 20 h for the formation of pendant MFCA–PPEI–EI via amidation. To obtain pendant MFCA–PPEI, unused amine units in MFCA–PPEI–EI were capped by propanoic acid using the same EDAC-catalyzed amidation reaction. For the capping, propanoic acid (0.4 mL, 5.3 mmol) was added to the reaction mixture containing MFCA–PPEI–EI, and the mixture was stirred at 0 ℃ for 12 h. Upon the removal of solvents, the reaction mixture was dissolved in chloroform and precipitated into hexane and dissolved in chloroform again and precipitated into acetonitrile. The polymer sample was then dissolved in water to form a brown aqueous solution. The solution was centrifuged vigorously to remove any unreacted MFCA, followed by dialysis against freshwater for 3 days to remove residual small molecules. After dialysis, water was removed to yield pendant MFCA-PPEI as a dark brown solid. The solid polymer sample was dried in a vacuum oven before characterizations.

MFCA–Diethylamine. EDAC (9.2 mg, 0.048 mmol) was added to a solution of MFCA (20 mg, 0.026 mmol) and diethylamine (6 μL, 0.06 mmol) in 12 mL of a chloroform–bromobenzene (3:1 v/v) mixture. After being stirred for 20 h, the reaction mixture was separated on a silica gel column using chloroform as eluent to yield MFCA–diethylamine as a dark solid (10 mg, 47% yield). The molecular structure of the compound was positively identified in NMR and MALDI-TOF MS analyses.[30]

Pendant MFPCA–PPEI. In a typical reaction, EDAC (14 mg, 0.073 mmol) was added to a solution of MFPCA (12 mg, 0.013 mmol) and PPEI–EI (102 mg, x_{NH} = 14%) in 5 mL of pyridine–chloroform (2:1 v/v) mixture. The coupling reaction was allowed to proceed for 48 h at room temperature. Then, propanoic acid (0.4 mL, 5.3 mmol) was added, and the reaction was allowed to proceed for an additional 16 h. Upon the removal of solvents and volatile components, the reaction mixture was dissolved in water. The brown aqueous solution was centrifuged vigorously to remove any unreacted MFPCA, followed by dialysis against freshwater for 3 days to remove residual small molecules. After dialysis, water was removed on a rotary evaporator to yield pendant MFPCA–PPEI as a dark solid. The solid polymer sample was dried in a vacuum oven at room temperature before characterizations.

MFPCA–Diethylamine. EDAC (9.2 mg, 0.048 mmol) was added to a solution of MFPCA (20 mg, 0.022 mmol) and diethylamine (6 μL, 0.06 mmol) in 6 mL of a pyridine–chloroform (2:1 v/v) mixture. The reaction was allowed to proceed for 24 h at room temperature. Upon the removal of solvents and volatile components on a rotary evaporator, the reaction mixture was separated on a silica gel column using chloroform as eluent to yield MFPCA–diethylamine as a dark solid. The solid sample was dried overnight under vacuum at 60 ℃ (11 mg, yield 42%). The molecular structure of the compound was positively identified in NMR and MALDI-TOF MS characterizations.[31]

Aqueous Solubility. Solubilities of the pendant polymer samples in room-temperature water were determined gravimetrically. In a typical experiment, 100 mg of pendant polymer sample was added to 1 mL of water. After sonication, the polymer–water mixture was centrifuged vigorously, followed by the removal of the clear aqueous solution. The residual polymer sample was dried, and its weight was determined and used in the solubility calculation.

Results and Discussion

PPEI and PPEI–EI polymers are highly water-soluble. In fact, the aqueous solution concentration of these polymers is limited more by viscosity than by solubility. With the covalent attachment of fullerenes, these polymers serve as carriers to introduce hydrophobic fullerene cages into water or polar solvents to form homogeneous solutions. For attaching fullerenes to the linear polymers in a structurally controllable fashion to preserve the fundamental properties of fullerene cages, the preparation of pendant fullerene–PPEI polymers represents an effective approach.

The pendant MFCA–PPEI was characterized in GPC analyses. With chloroform as mobile phase, however, significant tailing was observed in the chromatogram. Interactions beyond size exclusion were suspected as being responsible for the results. As reported recently,[32] fullerene-containing polymers in polar solvents often exhibit GPC behavior that is characteristic of polyelectrolytes, and the polyelectrolyte effects can be eliminated by adding lithium bromide salt to the mobile phase.[32] Thus, the pendant polymer was analyzed using DMSO with 0.2% lithium bromide as mobile phase. Under the GPC conditions, the tailing problem became negligible. The GPC analysis of the pendant MFCA–PPEI yielded an average molecular weight M_w of 49 000 in reference to poly(2-vinylpyridine) standards, which is comparable to that of the parent PPEI. The results suggest no cross-linking due to the attachment of MFCA to multiple PPEI polymer chains. The absence of cross-linking should be expected considering the specificity of the EDAC-catalyzed MFCA–PPEI–EI coupling reaction. Thus, the pendant fullerene polymer most likely has a "charm bracelet" type structure in which MFCA is attached to the linear PPEI chain via an amide linkage (Scheme 1).

Separately, MFCA–diethylamine as a monomeric model compound was prepared via the same EDAC-catalyzed amidation reaction (Scheme 1).

The UV–vis absorption spectrum of the pendant MFCA–PPEI is broader than those of free MFCA and the monomeric model compound MFCA–diethylamine (Figure 1). The sharp absorption peak at ~430 nm and the weak band at ~700 nm characteristic of MFCA and other methano-C_{60} derivatives become less pronounced in the spectrum of the polymer-bound MFCA. The UV–vis results were used to estimate the content of fullerene cages in the pendant polymer. Under a rough assumption that the total transition probability (integrated molar absorptivity) in the visible (400–750 nm) remains unchanged from the monomeric model compound to the pendant polymer, there are the following relationships.

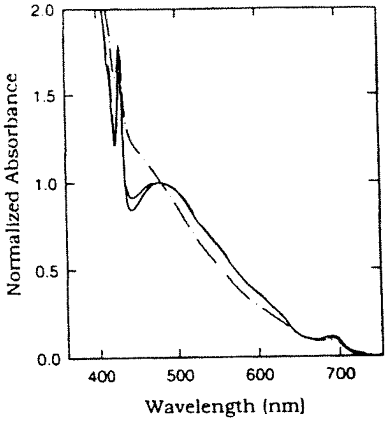
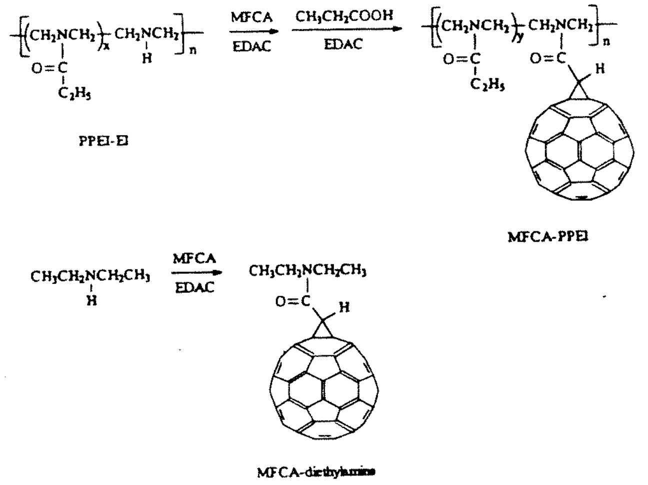


Figure 1. UV–vis absorption spectra of the pendant MFCA–PPEI in chloroform (- · -), the monomeric model compound MFCA–diethylamine in chloroform (- - -), and free MFCA in chloroform–DMSO (—).

Scheme 1



$$\text{transition probability} \propto \int(\epsilon/\lambda)\,d\lambda \approx (1/\lambda_{AVE})\int\epsilon\,d\lambda \quad (2)$$

where λ_{AVE} represents the average wavelength for the wavelength range. Thus,

$$\int\epsilon_{MFCA,P}\,d\lambda = \int\epsilon_{MFCA,M}\,d\lambda \quad (3)$$

$$\epsilon_{MFCA,P} = A_{MFCA,P}(x_{MFCA}C_{W,POLYMER})^{-1}M_{MFCA} \quad (4)$$

where the subscripts P and M denote polymer-bound and monomeric MFCA, $A_{MFCA,P}$ is the observed absorbance corresponding to the pendant polymer weight concentration $C_{W,POLYMER}$, and M_{MFCA} represents the MFCA molecular weight. Thus, the MFCA content in the pendant MFCA–PPEI can be calculated as follows.

$$x_{MFCA} = [\int(A_{MFCA,P}C_{W,POLYMER}{}^{-1}M_{MFCA})\,d\lambda]/[\int\epsilon_{MFCA,M}\,d\lambda] \quad (5)$$

The MFCA content in the polymer sample thus estimated is ~11% (wt/wt), which corresponds to the C_{60} cage content of ~10% (wt/wt) in the pendant MFCA–PPEI.

The FT-IR spectrum of the pendant MFCA−PPEI is rather similar to that of the parent PPEI, except for an additional weak absorption peak at ~527 cm^{-1} due to functionalized C_{60} cage in the polymer-bound MFCA. A similar absorption peak is in the FT-IR spectrum of the monomeric model compound MFCA−diethylamine. It should be noted that the IR absorption at ~527 cm^{-1} is characteristic of the C_{60} cage. The peak is one of the four signals in the FT-IR spectrum of C_{60} and is always preserved in the C_{60} cage functionalization. FT-IR spectra of different classes of C_{60} derivatives[33] and the C_{60} dimer[34] all show the peak at ~527 cm^{-1}.

The pendant MFCA−PPEI was characterized using ^{1}H and ^{13}C NMR methods for more direct structural information on the polymer. The ^{1}H NMR spectrum of the pendant MFCA−PPEI[35] is essentially the same as that of the parent PPEI, except for an additional weak and broad peak at ~4.6 ppm, which may be attributed to the methano proton in the polymer-bound MFCA. The same proton signal is at 5.1 ppm in the spectrum of free MFCA and 4.92 ppm in the spectrum of the monomeric model compound MFCA−diethylamine.[30] The ^{13}C NMR spectrum of the pendant MFCA−PPEI[36] is also similar to that of PPEI, but with weak peaks in the fullerene region. These somewhat broad fullerene peaks may be assigned to polymer-bound fullerene cages and used as evidence for the attachment of MFCA to PPEI. However, a more detailed analysis of the effects associated with the pendant polymer formation was hindered by the quality of the ^{13}C NMR spectrum. An improvement of the NMR signal quality for polymer-bound fullerene carbons was limited by the concentration of fullerene cages in the NMR solution. In principle, the concentration of fullerene cages may be increased by increasing the content of fullerene cages in the pendant polymer and/or the polymer solution concentration. In practice, however, the pendant polymer with a higher fullerene cage content is significantly less soluble, whereas the higher polymer concentration makes the solution too viscous for NMR measurements.

^{1}H NMR is certainly a more sensitive method, but it still has significant problems with the characterization of the pendant MFCA−PPEI. While the methano proton in MFCA may be used as a label, its signal at ~4.6 ppm in the spectrum of the pendant MFCA−PPEI is close to those broad and intense peaks of protons on the polymer backbone,[35] making it a less reliable proton label in the ^{1}H NMR characterization of the pendant polymer. As a result, a modification to the fullerene structure to include a functional group whose ^{1}H NMR signals do not overlap with those of PPEI was considered. Since there are no aromatic protons in PPEI, the methano-C_{60} derivative MFPCA with a phenyl group at the methano bridge was selected for the preparation of pendant MFPCA−PPEI polymer. The phenyl protons serve as labels for the polymer-bound fullerene in ^{1}H NMR characterization. With the carboxylic acid group in MFPCA farther away from the fullerene cage, it is more reactive, and its coupling reaction with the secondary amine units in PPEI−EI is more efficient and controllable. Similarly, MFPCA−diethylamine as a monomeric model compound was prepared in the same EDAC-catalyzed amidation reaction to simulate the formation of pendant MFPCA−PPEI (Scheme 2).

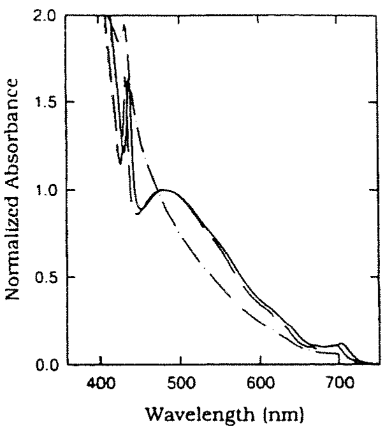
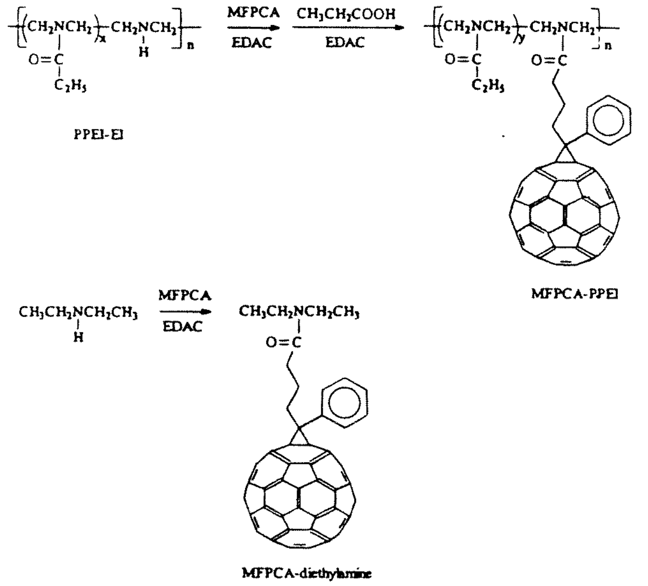


Figure 2. UV−vis absorption spectra of the pendant MFPCA−PPEI in chloroform (- · -), the monomeric model compound MFPCA−diethylamine in chloroform (- - -), and free MFPCA in CS_2 (—).

Scheme 2



The UV−vis absorption spectrum of the pendant MFPCA−PPEI is also broader than those of free MFPCA and the monomeric model compound MFPCA−diethylamine but retains some of the features that are characteristic of methano-C_{60} derivatives (Figure 2). Under the same rough assumption that the total transition probability in the visible (400−750 nm) remains unchanged from the monomeric model compound MFPCA−diethylamine to the pendant MFPCA−PPEI, a MFPCA content of 13% (wt/wt) in the polymer was estimated in terms of eq 5, which corresponds to a C_{60} cage content of 10.5% (wt/wt) in the pendant MFPCA−PPEI.

The ^{1}H NMR spectrum of the pendant MFPCA−PPEI in $CDCl_3$[37] is similar to that of the parent PPEI, except for the aromatic region. A comparison of the ^{1}H NMR spectra of the pendant polymer, the monomeric model compound MFPCA−diethylamine, and free MFPCA is shown in Figure 3. The aromatic proton signals due to the phenyl group in polymer-bound MFPCA are downfield shifted and significantly broader. The ^{1}H NMR spectrum of the pendant MFPCA−PPEI was also used to estimate the MFPCA content in the polymer. According to the integration of the aromatic proton signals in reference to that of the peak at 3.45 ppm ($NCOCH_2CH_3$), the MFPCA content of 14% (wt/wt) thus estimated, which corresponds to the C_{60} cage content of 11% (wt/wt), is in good agreement with the value estimated in

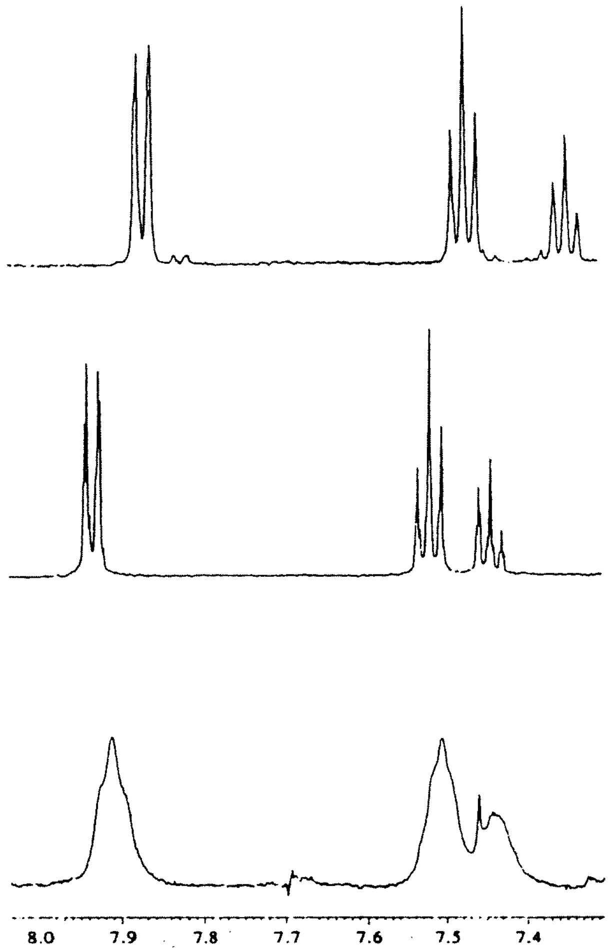


Figure 3. A comparison of the aromatic proton signals in the ^1H NMR spectra (500 MHz, TMS internal standard) of the pendant MFPCA–PPEI in $CDCl_3$ (bottom), the monomeric model compound MFPCA–diethylamine in $CS_2/CDCl_3$ (middle), and free MFPCA in CS_2 (top).

terms of eq 5 for the UV–vis absorption results. Such a good agreement is significant. While it may not validate the rough assumption that the total transition probability in the visible wavelength region is unchanged from the monomeric model compound to the pendant polymer, it suggests that the errors associated with the rough assumption may have fortuitously been canceled in the estimate of the fullerene content in MFPCA–PPEI. Thus, as long as the calculations (eqs 1–5) for MFCA–PPEI and MFPCA–PPEI are subject to similar errors, the rough estimate of the fullerene content in MFCA–PPEI in terms of the absorption results is probably not far from the correct value.

The ^{13}C NMR spectrum of the pendant MFPCA–PPEI[38] is again dominated by signals of the PPEI backbone, though there are clearly structured peaks in the fullerene region due to polymer-bound fullerene cages. The aromatic carbon signals due to the phenyl group in the polymer-bound MFPCA are also shifted from those of the monomeric model compound MFPCA–diethylamine and free MFPCA.

Both pendant MFCA–PPEI and MFPCA–PPEI polymers are soluble in water, forming dark colored aqueous solutions. The equivalent aqueous solubilities of C_{60} cages in the pendant MFCA–PPEI and MFPCA–PPEI are 9.5 and 10 mg/mL, respectively, which are significantly higher than the solubility of the parent C_{60} in room-temperature toluene (~3 mg/mL).[39] The pendant fullerene polymers are also highly soluble in chloroform and more polar solvents such as DMSO. The high equivalent aqueous solubilities of the polymer-bound C_{60} cages show that the pendant polymers are effective vehicles for the introduction of fullerenes into water or a hydrophilic environment.

The mole fraction of amine units in PPEI–EI has little effect on the preparation and properties of pendant fullerene–PPEI polymers. For the PPEI–EI copolymer samples of different x_{NH} values (3%, 7%, and 14%), their EDAC-catalyzed amidation reactions with MFCA and MFPCA yielded pendant polymers with similar properties.

The EDAC-catalyzed amidation reactions between methano-C_{60} carboxylic acids and the polymer-bound secondary amines are specific, forming most likely "charm bracelet" type pendant polymer structures. Because of the presence of amines, however, the possibility of thermal nucleophilic amine–fullerene addition as a competing reaction should be considered. In a control experiment, *tert*-butyl methano-C_{60} carboxylate was stirred with PPEI–EI under similar experimental conditions as those used for the EDAC-catalyzed amidation. No meaningful reaction between the carboxylate and PPEI–EI was observed. The results from the control experiment indicate that thermal nucleophilic amine–fullerene addition is insignificant in the solution containing methano-C_{60} cages and PPEI–EI polymers. In addition, the solution environment in the EDAC-catalyzed amidation is acidic, making the competing nucleophilic addition more difficult. The absence of nucleophilic amination of fullerene cages under the experimental conditions for EDAC-catalyzed amidation is consistent with the GPC results that suggest no meaningful cross-linking of linear PPEI chains in the pendant polymer samples.

In the pendant fullerene polymers prepared via the EDAC-catalyzed amidation, the fullerene cages are largely undisturbed. UV–vis absorption spectra of the polymer-bound methanofullerenes, though broader, retain the characteristic features observed in the spectra of monomeric methanofullerene derivatives. However, mechanistic details concerning the spectral changes upon the attachment of fullerene cages to the polymer remain to be understood. Since there are abundant amino groups in biological systems, the same carboxylic acid–amine coupling reaction may be used to introduce fullerene cages into biomacromolecular structures. In fact, it has been reported recently that methano-C_{60} dicarboxylic acids may be coupled to amino groups in the natural protein bovine serum albumin (BSA) to yield fullerene–protein conjugates.[12]

In summary, pendant MFCA–PPEI and MFPCA–PPEI were prepared via carbodiimide-catalyzed amidation reactions of methano-C_{60} carboxylic acids with polymer-bound secondary amine units. The pendant polymers were characterized by GPC, NMR, and other instrumental methods. The polymer structures are most likely of the "charm bracelet" type, without meaningful cross-linking of linear PPEI chains. With the pendant polymers being highly water-soluble, the equivalent aqueous solubilities of the polymer-bound fullerene cages are significantly higher than that of the parent C_{60} in toluene. Thus, the preparation of pendant fullerene polymers represents an effective approach for introducing fullerene cages into an aqueous solution or highly polar environment.

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Supporting Information Available: Synthetic procedures for MFCA and MFPCA. This material is available free of charge via the Internet at http://pubs.acs.org.

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(31) ^{1}H NMR (300 MHz, CS_2/C_6D_6): δ 4.91 (s, 1H), 3.98 (q, 2H), 3.68 (q, 2H), 1.59 (t, 3H), 1.33 (t, 3H) ppm. ^{13}C NMR (75 MHz, CS_2/C_6D_6): δ 164.11, 148.15, 147.27, 145.49, 145.35, 145.06, 145.05, 145.04, 144.55, 144.53, 144.53, 144.16, 143.84, 143.58, 143.21, 142.83, 142.65, 142.36, 143.16, 141.97, 141.05, 140.81, 139.19, 136.37, 72.03, 42.64, 41.56, 40.98, 14.83, 13.17 ppm. MALDI-TOF MS: 833.

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(34) ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.6 (bs, weak), 3.47 (bs, 2 × 2H), 2.40 (bs, 2H), 1.12 (bs, 3H) ppm.

(35) ^{13}C NMR (75 MHz, $CDCl_3$): δ 174.4, 173.8, 148−145 (weak), 46.9, 44.5, 25.8, 9.3 ppm.

(36) ^{1}H NMR (500 MHz, $CS_2/CDCl_3$): δ 7.94 (d, 2H), 7.54 (t, 2H), 7.45 (m, 1H), 3.38 (q, 2H), 3.29 (q, 2H), 2.95 (m, 2H), 2.52 (t, 2H), 2.23 (m, 2H), 1.16 (t, 3H), 1.13 (t, 3H) ppm. ^{13}C NMR (125 MHz, $CS_2/CDCl_3$): δ 171.2, 149.1, 148.1, 145.9, 145.3, 145.2, 145.0, 144.7, 144.6, 144.5, 144.4, 144.1, 143.8, 143.3, 143.2, 143.0, 142.5, 142.4, 142.3, 142.2, 141.2, 140.9, 138.1, 137.5, 137.2, 132.25, 128.49, 128.29, 80.2, 52.0, 42.0, 40.0, 36.0, 33.0, 22.0, 13.0, 12.0. MALDI-TOF MS: 951.

(37) ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.91 (bs, 2H), 7.51 (bs, 2H), 7.44 (bs, 1H), 3.42 (bs, 2 × 2H), 2.3 (bs, 2H), 1.1 (bs, 3H) ppm.

(38) ^{13}C NMR (125 MHz, $CDCl_3$): δ 175, 174.6, 145−141 (weak), 132.0, 128.6, 128.5, 46, 43.5, 26, 9.5 ppm.

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2

Molecular Basis of Bacterial Adhesion

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I. INTRODUCTION

Bacterial infections are responsible for a broad spectrum of human illnesses and medical device complications. For example, urinary tract infections caused by *Escherichia coli* affect over 7 million people annually and are among the most common infectious diseases acquired by humans.[39] Enteropathogenic *E. coli* (EPEC) and shiga toxin-producing *E. coli* (STIC) are diarrhoegenic pathogens causing serious health problems in both industrialized and developing countries.[26,15] *Helicobacter pylori* have been found to be a main factor in the development of gastric and duodenal ulcers and are believed to be a causitive factor of gastic cancer.[34] *Staphylococcus aureus* and *Staphylococcus epidermidis* are major causes of infections associated with wounds, indwelling catheters, and cardiovascular and orthopedic implant devices.[1,19,24,25,35,49,56,59]

Bacteria have a strong tendency to attach to surfaces. Attached cells will form a colony (biofilm) consisting of prokaryotic cells, surrounded by a matrix of biomolecules secreted by the cells. Although the structure and functions of biofilms are as varying as the type of bacteria, the same four step process is always followed in the creation of the biofilm.[21,57]

During the first step, a series of small molecules (initially water and salt ions) will adsorb to the surface. Subsequently, the substrate surface will be covered with a single layer of small organic molecules or proteins that are present in the medium. The mixture of water, ions and proteins is called conditioning film and is always present before the first microorganisms arrive at the surface.[17,50]

The second step is characterized by the initially reversible adsorption of microorganisms to the conditioning film. The microbes arrive by Brownian motion, gravitation, diffusion, or intrinsic motility. They may also adhere to each other forming microbial aggregates before adsorbing to the conditioning film. Since the microorganisms adhere to the conditioning film and not the surface itself, the strength of the initial biofilm depends on the structure of the conditioning film.[8,29]

The initially reversible adsorption becomes irreversible, mainly through the secretion of exopolymeric substances by the adsorbed microorganisms in step three.[18,40] These substances will incorporate in the conditioning film and strengthen its cohesiveness. In a

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few cases, an entirely microbially derived conditioning film has been observed.[7] Once bound to the surface, many bacterial strains, including *S. aureus* and *S. epidermidis*, additionally form a polysaccharide based biofilm surrounding the bacterial colony. Biofilm effectively inhibits phagocytosis and makes the contained bacteria impervious to antibiotics, thus making implant removal an essential part of treatment once an infection is established.[12,24,25,35,56]

Finally, the number of microorganisms in the biofilm accumulates mainly through *in situ* cell growth. The final structure and composition of the biofilm is determined by these initial events. Other aspects such as the influence of surface active compounds secreted by the microorganisms,[41] the hydrodynamic environment,[48,56] the surface roughness,[5,46] the available nutrients,[6,22] and the attraction and adhesion to other microorganisms from the surrounding medium[4,20,32,33] are thought to be of secondary importance regarding the final outcome of the biofilm.[9]

Bacterial adhesion leading to infection can be divided into three distinct categories: specific adhesion to host cell surface molecules, specific adhesion to extracellular matrix and blood plasma derived molecules, and adhesion to biomaterial surfaces of medical devices. In this chapter, an overview of the current understanding of the molecular basis of bacterial adhesion as it pertains to each of the three categories of bacterial adhesion is presented, followed by modeling of bacterial adhesion based upon the general principles governing molecular adhesion. Particular emphasis is given to interactions between the initially arriving microorganisms and the conditioning film at the molecular level.

II. MICROBIAL ADHESION TO EXTRACELLULAR MATRIX MOLECULES

The interactions of arriving microorganisms with a conditioning film on a surface are usually mediated by specific binding events between adhesins on the microbe surface and receptors of the extracellular matrix (ECM). Receptor binding may subsequently activate a series of complex signal transduction cascades in the host cell, which may be either inhibitive or beneficial to bacterial invasion. In several bacterial species, including *E. coli*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, and *Salmonella enteritidis*, adhesins are presented at the tips of complex cell-surface structures which extend from the outer cell membrane called pili or fimbriae.[52] Pili are classified as P, type 1, type IV, and curli, each with distinct structural organization and assembly mechanisms. Alternatively, nonpilus adhesins may be directly presented from the bacterial cell surface as well.[52]

The molecular basis for bacterial adhesion to ECM molecules has been widely studied, and found to occur through specific binding mechanisms involving both piliated and nonpilated bacterial adhesins. These processes involve integrins which are a family of heterodimeric ($\alpha\beta$) cell-surface receptors that recognize specific ECM submolecular structures.[43] While much remains to be learned of the specific molecular mechanisms involved, bacterial cells have been found to utilize many of these same cell wall receptors to specifically adhere to ECM molecules, including fibronectin, collagens, laminin, vitronectin, thrombospodin, elastin, bone sialoprotein and GAGs like heparin, heparan sulphate, and chondroitin sulphate.[34] Bacterial adhesins, which bind with ECM, are termed microbial surface components recognizing adhesive matrix molecules (MSCRAMM).[37,43,44]

One of the most widely studied systems of bacteria-ECM interaction is *S. aureus* binding to fibrinogen. Fibrinogen is specifically recognized by several host cell integrins, including the platelet integrin $\alpha_{IIb}\beta_3$.[43] Two of the most widely understood fibrinogen binding proteins expressed by *S. aureus* strains are called clumping factors A and B (ClfA and ClfB). Studies have indicated that ClfA binds exclusively with the γ-chain of fibrinogen, while ClfB binds to both α- and β-chains.[19]

ClfA is a 933 residue protein which includes a 520 residue region that contains its fibrinogen binding domain. This binding domain is preceded by a very interesting molecular structure consisting of 154 repeats of a serine-aspartate dipeptide sequence.[19] At a physiologic pH of 7.4, the carboxylic acid side groups of the aspartic acid residues will be deprotonated (thus carrying a single negative charge on each residue) and the interdispersed hydroxyl side groups of the serine residues will be strongly hydrophilic. The repulsion of the sequential aspartic acid residues coupled with the hydrophilicity of the serine residues should thus provide a large electrostatic driving force to extend the adhesin outward from the bacterial cell surface, much like hairs standing up on one's head when electrostatically charged.

Studies suggest that ClfA binds to two distinct sites in the γ-chain of fibrinogen by molecular structures very similar to the fibrinogen-binding integrin, $\alpha_{IIb}\beta_3$. ClfA and the platelet receptor $\alpha_{IIb}\beta_3$ have been found to recognize the same 400-411 residue section at the extreme C-terminus of the fibrinogen γ-chain (residue sequence ...HHLGGA-KQAGDV), and studies have shown that alteration of only the last four residues (... AGDV) is sufficient to inhibit ClfA binding.[37,19] The γ-chain binding site of ClfA has been mapped to a region of the α_{IIb} polypeptide of the $\alpha_{IIb}\beta_3$ integrin and both contain the Ca^{2+} binding EF-hand motif found in many eukaryotic calcium ion binding proteins. The EF-hand motif consists of about 12 residues, with the proposed ClfA sequence being **D**S**D**G**N**VIY**T**F**TD**, which represents residue numbers 310-321 in the protein. The sequence letters indicated in bold are the residues specifically involved with cation binding. These residues form a coordination sphere for the divalent cation and are flanked by α-helices which provide support structure for this motif to maintain proper functional conformation.

Studies have shown that high concentrations of calcium ions inhibits ClfA-fibrinogen binding in a manner similar to that observed for integrin-ligand binding.[19,43] Thus, calcium ion concentration serves as an important regulator of *S. aureus* binding to fibrinogen. The second fibrinogen binding site of ClfA, which is also the $\alpha_M\beta_2$ integrin binding site, involves fibrinogen γ-chain residues 190-202. Similar to $\alpha_M\beta_2$, this ClfA binding site also includes a cation-binding metal ion-dependent adhesion site (MIDAS) motif.[19] The ClfB protein does not have an EF hand-like tertiary structure, however, it does possess a MIDAS-like motif, such that the binding of both ClfA and ClfB to their fibrinogen ligand sites are regulated by Ca^{2+} concentration. The specific binding sequences in ClfB and their role in ligand binding have not yet been elucidated to the extent of ClfA.[19]

S. aureus also has been found to express MSCRAMM adhesins for both fibronectin (FnbpA and FnbpB) and collagen.[13,34] Studies have shown that there are at least two binding sites on fibronectin, one occurring in its N-terminus domain, and the other in its C-terminus domain.[34] Although the molecular details of these interactions have not been as widely reported in the literature as the fibinogen binding system, the actual structure of an *S. aureus* collagen-binding adhesin has been determined and is available in the Brookhaven Protein Data Base under protein code 1AMX.

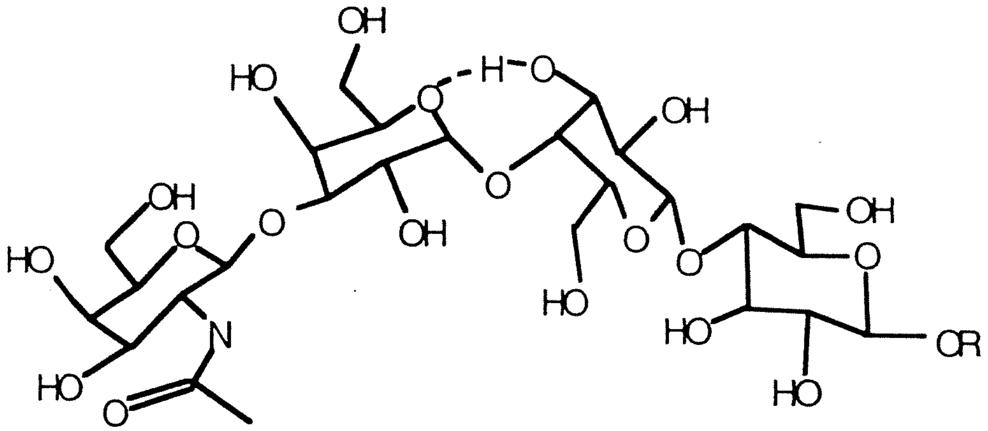


Figure 1. Molecular structure of the globotetraose-binding site of a class II PapG. Oxygen and hydroxyl groups indicated in bold are believed to form hydrogen bonds with the adhesin. Adaped from Striker et al.[53]

III. HOST CELL ADHESION MECHANISMS

Bacterial adhesion to host cells of the urinary tract has been found to occur by specific molecular interactions between adhesins located on the distal tip of pili extending from the bacterial outer membrane and receptor molecular structures present on the host cell outer surface. Although the exact molecular structures involved for many of these interaction are yet to be determined, the specific binding mechanisms involved in a few systems have been relatively well characterized.

Uropathogenic *E. coli* has been shown to adhere to erythrocytes and uroepithelial cells of the kidney and urinary tract.[28,53] Studies have revealed that this specific binding event occurs between PapG adhesins located at the tip of P pili and Galα(1-4)Gal saccharide epitopes in the globo series of glycolipids. This saccharide structure has been determined to be linked by a β-glucose residue to a ceramide group anchoring the receptor in the host cell membrane. The receptor-binding domain of PapG has been determined to lie in the N-terminus of the protein. An example of the globotetraose-binding site of a class II PapG is presented in Figure 1. Studies involving sequential functional group replacement in Galα(1-4)Gal have revealed that PapG binds to this receptor, in part, by hydrogen binding with a series of five oxygen atoms located along the edge of the disaccharide surrounding a central hydrophobic core.[28]

Uropathogenic *E. coli* expressing type-1 pili tipped with the FimH adhesin (a 30-kDa protein[51]) have been shown to specifically bind to mannosylated integral membrane glycoproteins (uroplakins) presented from the luminal surface of bladder epithelial cells using a murine cystitis model.[39] This bacteria exhibits a very interesting but not yet fully understood mechanism to facilitate close bacteria–host cell interactions. Host cell contacting pili were found to be only 0.12 μm in length in contrast to the typical 1-2 μm length for non-contacting pili. This suggests a possible pili retraction mechanism to enhance tight bacterial–host cell apposition, with subsequent possible host-cell internalization of the *E. coli*[39] Sokurenko and coworkers[51] investigated the differences in the 300 residue sequence of FimH and their respective adhesive characteristics for fourteen *E. coli* strains. This study revealed that the FimH sequences where essentially homologous to one another except for 2 specific residue changes involving a swapping of arginine and serine residues at positions 70 and 78. The exchange of these two residues was found to result in distinct differences in bacterial adhesion. Although not well understood, this alteration is believed to influence the structure of the saccharide binding pocket in FimH for mannose binding.[51]

A different bacterial mechanism has been found to occur for the mucosal lining of the intestine leading to microvilli effacement and diarrhea. A four stage infection process has been suggested involving initial attachment of enteropathogenic *E. coli* bacteria (EPEC) to the microvilli enterocyte cell surface:

1. A nonpiliated adhesin mechanism;
2. Type III bacterial secretion of 80kDa proteins (*E. coli* secreted protein, EspE) which mediate cytoskeleton disruption and the formation of tyrosine-phosphorylated translocated intimin receptors (TIR) on the host-cell surface for intimin binding;
3. Intimin-binding mediated bacterial attachment to the intestinal mucosa; and
4. Bundle-forming pili (BFP) mediated bacterial colonization.[15,26]

Others have suggested that BFP serves as the adhesin controlling initial host cell contact as well as bacterial colonization.[2]

S. aureus can bind to endothelial cells through its fibrinogen binding clumping factors ClfA and ClfB. Adhesion studies found that the preferential attachment of *S. aureus* to umbilical vein endothelial cells is mediated by fibrinogen adsorbed from plasma. Antifibrinogen antibodies could block the binding, indicating the specificity. Cheung et al. found that fibrinogen acts as a bridging molecule, attaching to both endothelial and *S. aureus* cell-wall integrins with each of its two γ-chains.[11]

Finally, some bacteria use the integrin on endothelial cells to invade the host. Filamentous hemagglutinin (FHA), an adhesin formed as a 50 nm monomeric rigid rod of *Bordetella pertussis*, interacts with two classes of molecules on macrophages, galactose containing glycoconjugates and the $\alpha_M\beta_2$ integrin which binds to the Arg-Gly-Asp (RGD) sequence in FHA.[47] Intimin, the outer membrane protein of *E. coli* also binds specifically to $\alpha_M\beta_2$ integrins and is inhibited by RGD containing peptides.[23]

IV. ADHESION TO BIOPOLYMERS AND BIOMATERIALS

A. Natural Biopolymers

In natural heterogeneous microbial ecosystems (such as soil or an aquatic environment), adherence of a cell to a solid surface confers several competitive advantages. The ability to bind to a solid biopolymer (such as the cellulose fiber to which the filamentous bacterium, *Thermomonospora curvata*, has bound itself in Figure 2) provides the cells with a reliable constant carbon and energy source.[27] The adhesion not only brings its surface bound enzymes (cellulosomes[31]) into intimate contact with the substrate, but also affords it prime access to whatever soluble depolymerization products are released by their catalytic action. Cellulosomal organization and molecular structure of its complex components has been most extensively studied in the mesophilic anaerobe. *Clostridium cellulovorans*. Its cellulosome is composed of three major subunits: as caffolding protein, an endoglucanase, and an exoglucanase.[16] The binding of cellulolytic microbes such as *Clostridium cellulovorans* to cellulose-containing substrate surfaces is

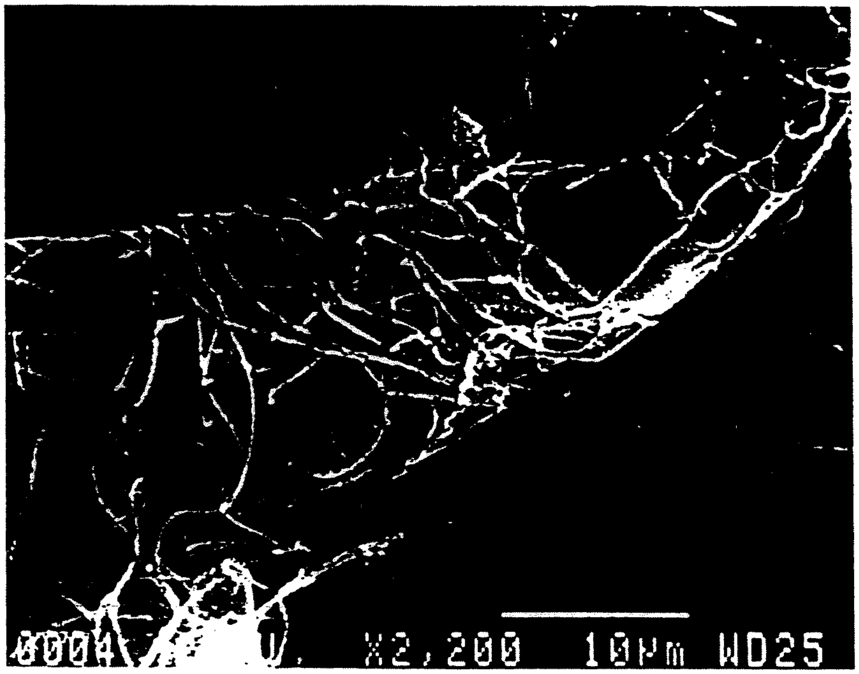


Figure 2. Filamentous bacterium *Thermonospora curvata* bound to cellulose fiber.

mediated by one or more of a heterologous group of cell surface-bound proteins containing cellulose binding domains (CBD). These CBDs have been classified into 10 families (I-X) on the basis of amino acid sequence homology.[55] The amino acid sequences of CBDs in *C. cellulovorans* and *C. josui* show high homology with those from other cellulolytic genera such as *Bacillus*. CBDs in this family contain several highly conserved amino acid sequences:[30]

1. Tryptophane-asparate-phenylalanine-asparagine-asparate-glycine-threonine
2. Isoleucine-alanine-alanine-isoleucine-proline-glutamine
3. Isoleucine-leucine-phenylalanine-valine-glycine

The cell surface-bound cellulosome in *Clostridium* species and in others is a complex of adherence and catalytic proteins. A major cellulosomal subunit (EngE) has been recently characterized.[54] EngE is anchored via a protein having triple-repeated surface layer homology (SLH) domains at the N-terminus; these domains appear to integrate into the lattice of the cell surface peptidoglycan-surface protein complex; they also bind with hydrophobic domains of the EngE. Therefore the cellulosic surface adhesion architecture in *C. cellulovorans* consists of catalytic units which have specific cellulose-binding domains, hydrophobic domains which act as linkers between the catalytic units and the SLH domains, and the SLH which integrates into the peptidoglycan-teichoic acid-lipoteichoic acid lattice which is the major structural component of the bacterial cell wall.[45] This attachment structure appears similar to that complexed with other surface-bound exoenzymes in related bacteria.[38]

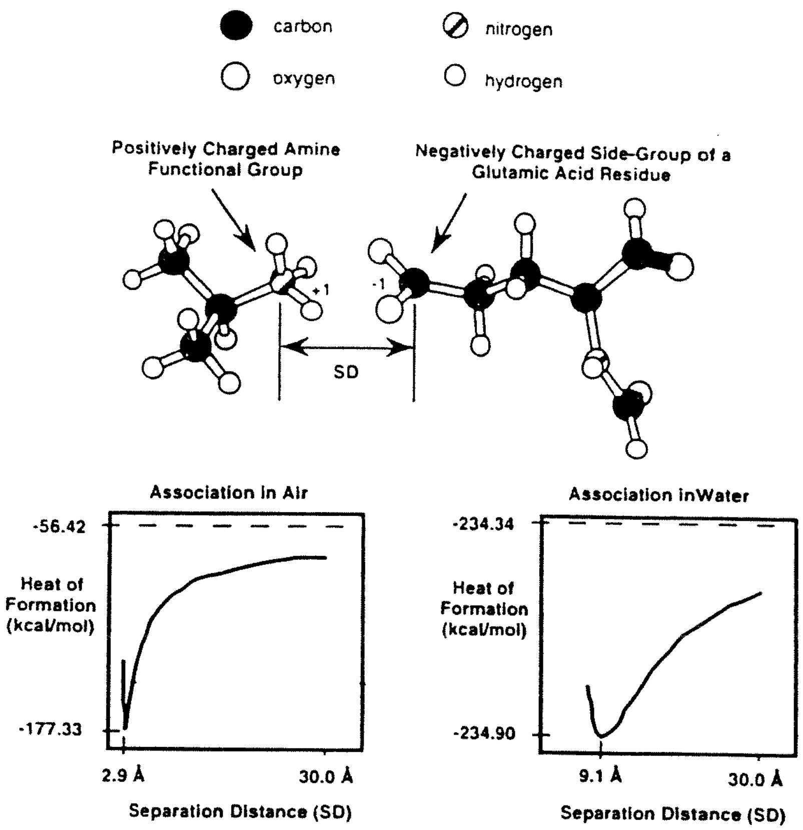
B. Synthetic Biomaterials

In vivo, coagulase-negative staphylococcal (CNS) (in particular *S. epidermidis*) and *S. aureus* are the leading causes of infection for body fluid-contacting medical devices, including intravascular catheters, cerebrospinal fluid shunts, prosthetic artificial heart valves, orthopedic devices, cardiac pacemakers, chronic peritoneal dialysis catheters, and vascular grafts.[19,25,35,49,58,59]

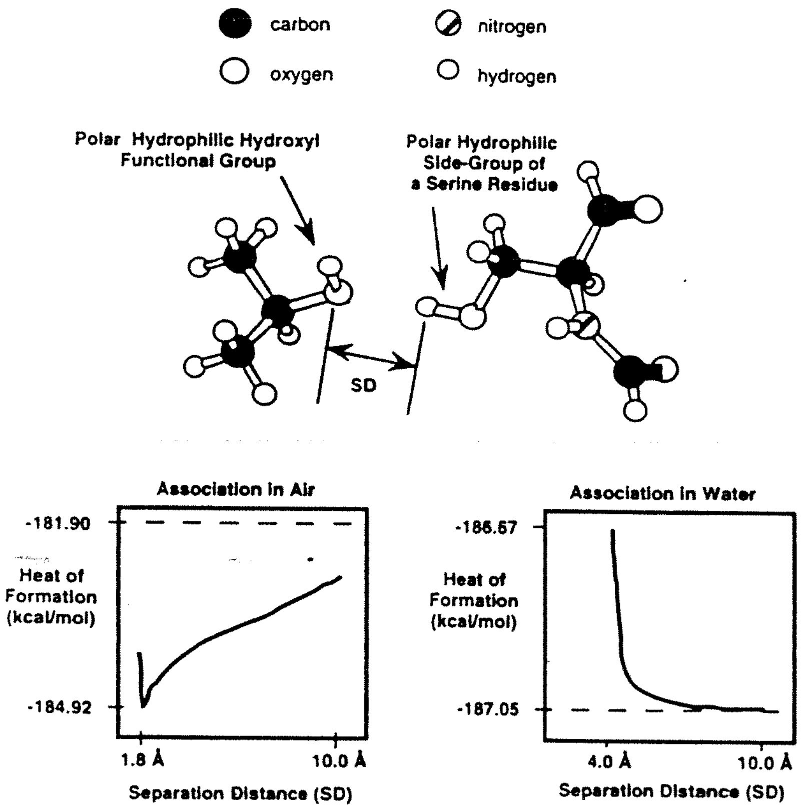
Serum proteins, such as albumin, fibronectin, fibrinogen and laminin, have been found to readily adsorb nonspecificially to biomaterial surfaces following body fluid exposure.[41] These adsorbed proteins form the conditioning film onto which specific bacterial adhesion takes place in mechanisms very similar to those that govern bacterial adhesion to extracellular matrix components.[1,34] An additional factor unique to biomaterials, however, is that the serum protein adsorption process may abnormally denaturate the proteins structure, thus potentially exposing binding sites not normally present in either the soluble or ECM form of the proteins. Fibrinogen is one of the major plasma proteins adsorbed onto implanted biomaterials and the adhesion of *S. aureus* to adsorbed fibrinogen and fibrin is an important initiator of infection[13,19,24] The specific adhesion mechanisms between bacteria and surface bound fibrinogen/fibrin are believed to be essentially the same as those which occur for bacteria adhesion to fibrinogen as an ECM component and have been addressed in the previous section.

Fibronectin is another predominant glycoprotein component of plasma which is incorporated into the fibrin matrix during blood clot formation and which also readily adsorbs to implant surfaces following blood contact.[59] This macromolecule is a dimeric glycoprotein composed of a series of domains comprising different combinations of type I, type II, and type III modules.[24] *S. aureus* has been shown to specifically bind to both soluble and adsorbed fibronectin, but has little affinity for fibrinogen incorporated into thrombi by itself without preexposure of the bacteria to soluble fibronectin.[59] Binding has been found to involve at least two *S. aureus* MSCRAMM fibronectin-binding proteins, known as FnbpA and FnbpB, and at least two binding sites on fibronectin involving both the N- and C-termini.[24,34] FnbpA and FnbpB are identical to one another apart from their N-terminal regions which is only 45% homologous.[13,24] In contrast to this, coagulase negative staphylococcus (CNS) does not bind soluble fibronectin; however, it exhibits significant affinity for the N-terminus domain of fibronectin incorporated into fibrin thrombi or immobilized on plastic surfaces.[34,59] These differences between soluble and bound fibronectin are believed to reflect conformational changes induced by adsorption; although soluble fibronectin has a globular structure, the two arms of the dimer are believed to unfold upon binding to expose previously hidden binding sites.[59]

Another pathway for bacterial infection involves the secretion of exopolymeric substances following initial bacterial adhesion to implant surfaces. *S. epidermidis*, for example, colonizes within a self-generated viscous biofilm largely composed of polysaccharides. This amorphous extracellular matrix substance enhances bacterial adhesion to biomaterials surfaces and provides the bacterial colony with protection from antibiotics and phagocytoses.[1,35,49,53] The process of colonization and biofilm production by *S. epidermidis* involves several key molecular interactions. *S. epidermidis* strains produce two similar insoluble polysaccharides, termed polysaccharide adhesins (PS/A) and polylsaccharide intercellular adhesins (PIA). PS/A production is generally correlated with bacterial adherence to biomaterial surfaces, apparently by nonspecific adsorption mechanisms, with subsequent biofilm formation and bacterial colonizaton mediated by PIA.[38] PS/A is a high-molecular-weight variably N-succinylated (65-100%) β-1,6-linked N


carbon
nitrogen
oxygen
hydrogen
Positively Charged Amine Functional Group
Negatively Charged Side-Group of a Glutamic Acid Residue
+1
-1
SD
Association in Air
-56.42
Heat of Formation (kcal/mol)
-177.33
2.9 Å
30.0 Å
Separation Distance (SD)
Association inWater
-234.34
Heat of Formation (kcal/mol)
-234.90
9.1 Å
30.0 Å
Separation Distance (SD)

(A)


carbon
nitrogen
oxygen
hydrogen
Polar Hydrophilic Hydroxyl Functional Group
Polar Hydrophilic Side-Group of a Serine Residue
SD
Association in Air
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Heat of Formation (kcal/mol)
-184.92
1.8 Å
10.0 Å
Separation Distance (SD)
Association in Water
-186.67
Heat of Formation (kcal/mol)
-187.05
4.0 Å
10.0 Å
Separation Distance (SD)

(B)

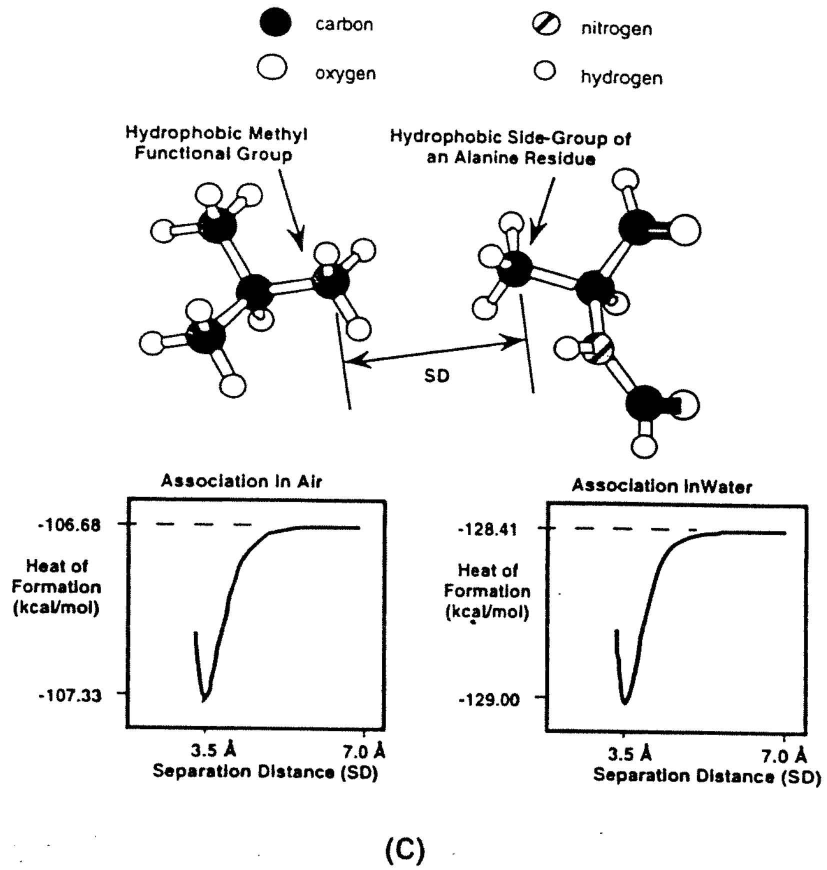

Figure 3. Molecular models illustrating intermolecular interactions as a function of chemical structure and environment. Plots present calculated heats of formation versus functional group separation distance (SD) in air and water. Dotted lines indicate energy at infinite separation. A) Ionic interaction between a positively charged amine functional group and a negatively charged carboxylic acid side-group of a glutamic acid residue. B) Polar interaction (hydrogen bonding) between a hydrophilic hydroxyl functional group and a hydrophilic hydroxyl side-group of a serine residue. C) Nonpolar interaction between a hydrophobic methyl functional group and a hydrophobic methyl side-group of an alanine residue. (Calculations conducted using semi-empirical quantum mechanical theory using MOPAC/PM3 with COSMO water simulation; CAChe Software, Oxford Molecular, Beaverton, OR.)

acetylated glucosamine.[38] NMR data suggests that PIA has an unbranched structure with β-1,6 interglycosidic linkages containing at least 130 residues. PIA is reportedly made up two primary types of polysaccharides, termed PS I (80%) and PS II (20%). PS I has 80 to 85% *N*-acetyl-D-glucosaminyl residues, with the remainder being non-*N*-acetylated positively charged D-glucosaminyl residues which are apparently randomly distributed along the chain. PS II is similar to PS I, however, it contains fewer non-*N*-acetylated side groups and a small number of phosphate and ester-linked succinyl residues, giving the overall chain a moderately anionic character.[25,35,49] The unbranched, long-chain structure of PS I and PS II, combined with their distributed anionic and cationic charges, is believed to be important for both intercellular adhesion and biofilm adherence, with van der Waals, electrostatic, and hydrogen bonding mechanisms providing molecular attraction between polysaccharide chains.[35]

Recent studies have found some evidence for cell-to-cell signaling in bacterial biofilms adsorbed to surfaces such as in the growth of *Pseudomonas aeruginosa* adsorbed onto glass.[14] Cell-to-cell signaling in bacterial biofilms may alter antibiotic resistance or adherence characteristics of other pathogens on medical implant surfaces.

V. MODELING MICROBIAL ADHESION

Molecular adhesion involves noncovalent intermolecular interactions governed by either secondary bonding between induced dipolar and dipolar functional groups (also referred to as physical bonding or van der Waals bonding) or electrostatic interactions between charged functional groups.[10] Although individual secondary bonds and electrostatic interactions within an electrolyte solvent (i.e., physiologic saline) are relatively weak compared to primary chemical bonds, their combined effects can result in very strong binding between macromolecules. The reader is referred to Chapter 1 for a review of intermolecular interactions.

These three basic types of secondary bonding and ionic interactions are graphically presented in Figure 3, which presents molecular models of 3 sets of molecules illustrating hydrophobic, neutral hydrophilic and ionic interactions. Two energy *vs* distance plots are presented for each model; one representing the calculated system heat of formation versus molecular separation in a solvent free environment (i.e., air) and the other in an aqueous solution. The net adsorption enthalpy for each set of molecules represents the difference in the heats of formation between the molecules at infinite separation compared to the system heat of formation when they are closely associated. From these energy plots, the hydrophobic functional groups are shown to only weakly attract one another over a short range of only about 6 Å in both the air and aqueous environments. In an aqueous environment, however, additional effects due to the entropy associated with the orientation of water molecules surrounding the functional groups provide an even larger thermodynamic driving force to minimize the solvent accessible surface, thus tending to strongly bind hydrophobic groups together. In contrast to this, interaction between the polar hydrophilic hydroxyl groups is shown to provide a relatively strong short-range molecular attraction in air but an effective energy barrier to binding in an aqueous environment due to preferential adsorption of water. Finally, interactions between positive and negative charges in an ionic system show very long-range strong attraction in air with close ion group association in the bound state. However, in an aqueous environment the ionic functional groups show long-range but relatively weak attraction with the low energy binding state, predicting that the functional groups are separated by a distance approximately equal to two water molecules, thus representing a solvation layer about each charged group.

These results have recently been measured using atomic force microscopy (AFM). The interactions between uncharged glycolipids with varying head-groups and the organic surfaces of self-assembled monolayers (SAMs) were measured in deionized water. It was found that in the absence of ions, the water structure surrounding the organic layers influences the adhesion. The presence of Ca^{2+} ions produced an enhanced attraction between sialic acid and the ganglioside GM1.[3]

VI. CONCLUDING REMARKS

As discussed above, the critical first step in the development of bacterial infections of tissues and medical devices involves bacterial adhesion to host cells, ECM glycoproteins, or biomaterials surfaces. These binding mechanisms most often involve very specific submolecular interactions, many of which are still not well understood. Therapies which can prevent or disrupt these molecular mechanisms have great potential for development as alternatives to antibiotics for the prevention and treatment of infection, thus emphasizing the importance of research directed toward the elucidation of the molecular basis for bacterial adhesion.

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Handbook of Bacterial Adhesion

Principles, Methods, and Applications

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III. KEY PERSONNEL

III.1. Roles, Responsibilities, and Time Commitment

Role and Time Commitment of Dr. Fred Stutzenberger (Co P.I.)
In addition to his overall supervisory role as Co P.I. on this project, Dr. Stutzenberger will actively participate in the in the culturing and viability determinations of bacterial cell-nanoparticle aggregates as described in Materials and Methods. Dr. Stutzenberger will commit approximately one-third of each summer session plus a proportional amount of time during the regular academic year.

Role and Time Commitment of Dr. Robert Latour (Co P.I.)
Dr. Latour will serve an overall supervisory roll for this whole program and in additon will work with Dr. Sun for the application of bioactive molecule functionality on the nanoparticles. He will also be responsible to direct the in vitro and the in vivo exposure sensitivity studies. Dr. Stutzenberger will commit approximately one-third of each summer session plus a proportional amount of time during the regular academic year.

Role and Time Commitment of Dr. Ya-Ping Sun (Co-Investigator)
Dr. Sun will be directly responsible for all aspects of the program related to nanoparticle synthesis. This will involve the development of the initial synthesis routes and the production of the designed nanoparticles throughout the full three year period of the program. Dr. Stutzenberger will commit approximately one-third of each summer session plus a proportional amount of time during the regular academic year although no salary support is requested for his efforts.

Role and Time Commitment of Dr. Tzuen-Rong Tzeng (Co-Investigator)
Dr. Tzeng will participate in the in-vitro study of *Campylobacter jejuni* including culturing and viability determinations of bacterial cell-nanoparticle aggregates as described in Materials and Methods. Dr. Tzeng will commit approximately one-twelfth (year one) and one-six (year two and three) of time during the regular academic year.

Role and Time Commitment of Dr. John Rodgers (Co-Investigator)
Dr. Rodgers will assist with the supervision of the in vivo exposure sensitivity studies in years 2 and 3 of the program. Time commitment for this work will be approximately one month per year.

Role and Time Commitment of Dr. Jesse Grimes (Co-Investigator via Subcontract)
Dr. Grimes be directly responsible for all of the in vivo bioactivity studies performed at NC State. He time commitment will be approximately one-third of each summer session plus a proportional amount of time during the regular academic year.

III.2. Vitae of Key Personnel

I. **Name: Fred J. Stutzenberger**
Date of Birth: November 10, 1940

II. A. **Academic training:**

B.S. (Biology), Bellarmine College, Louisville, KY (1962)
M.S. (Microbiology), Univ. Houston, Houston, TX (1964)
Ph.D. (Microbiology), Michigan State Univ., E. Lansing, MI (1967)
Post-doctoral training: Ministry of Agriculture Research Centre, Hamilton, New Zealand (1971-74)

B. **Employment history:**

1979-present:	Professor of Microbiology, Clemson University
1996:	Senior Research Fellow, Child Health Research Institute, Women's & Children's Hospital, Adelaide, Australia
1984-1985:	Research Fellow, Ministry of Fisheries and Agriculture, Ruakura Agricultural Research Centre, Hamilton, New Zealand
1975-1979:	Associate Professor, Clemson University
1974-1975:	Visiting Asst. Professor, Clemson University
1971-1974:	Research Fellow, New Zealand Ministry of Agriculture Research Centre, Hamilton, N.Z.
1969-1971:	Asst. Professor, Weber State Univ., Ogden, Utah
1967-1969:	Senior Microbiologist, National Center for Urban and Industrial Health, U.S. Public Health Service, Cincinnati, OH
1964-1967:	Research Assistant, Michigan State University
1962-1964:	Research Assistant, University of Houston

C. **Professional Organizations**

American Society for Microbiology
Society for Applied Bacteriology
Sigma Xi (life member)

D. **Current/Past Professional Positions**

Senior Editor, Letters in Applied Microbiology (1989-97)
Editorial Board, Journal of Industrial Microbiology (1994-)
Editorial Board, Applied Microbiology & Biotechnology(1996-)
Editorial Board, Enzyme and Microbial Technology (2000-)

E. Honors and Awards

Who's Who in American Colleges and Universities, 1961-2
Sigma Xi Outstanding Research Award, 1966
Michigan State University Microbiology Department Graduate Student Award for 1967
Clemson University Provost Research Award, 1988
Clemson University Community Service Award, 1993
Clemson University Alumni Distinguished Professor Nominee from College of Agriculture, Forestry and Life Sciences, 1996, 1998
Clemson University Faculty Innovation Award (co-recipient with Dr. Michael Kilgore, GHS/CU Biomedical Cooperative)

III. Refereed_Publications during past 4 years

IV.

Busch, J. E., E. G. Porter and F. Stutzenberger. 1997. Amylase induction and repression in *Thermomonospora curvata* during growth in maltooligosaccharides. J. Appl. Bacteriol. **82:669-676.**

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Stutzenberger, F.J. 1998. Composting as a biological model. In: *Instructional Resource, Unit one, Biology, A Comm;unity Context.* (W.H. Leonard and J.E.Penick, eds.), p. 12. International Thomson Publishing Co., Cincinnati, OH.

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Grubel, P., L. Huang, F. Stutzenberger *et al.*. 1998. Detection of *Helicobacter pylori* DNA in houseflies (*Musca domestica*) on three continents. The Lancet 352:788-789.

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Stutzenberger, F. 2000. Pectinases: Production, Properties and Applications. In: Encyclopedia of Microbiology, vol. 3, 2nd ed. (J. Lederberg, ed.), pp. 30-47, Academic Press, San Diego.

Riley, M.B., I. Collins, Y. Richardson and F. Stutzenberger. 2000 Extraction of fungal fatty acids for gas chromatography analysis. Mycologia **92**: 301-304.

Li, S. and F. Stutzenberger. 2000. The housefly (*Musca domestica*) as a possible vector for *Helicobacter pylori* at agricultural sites. Int. J. Environ. Health Res. **10:** 141-152.

Boland, T., F. Stutzenberger and R.A. Latour, Jr. 2000. The molecular basis of bacterial adhesion. In: *Handbook of Microbial Adhesion: Principles, Methods and Applications*, pp.21-49. (Y.H.An and R.J. Friedman, eds), Humana Press, Totowa, NJ.

In Press (accepted for publication as of May 30, 2000)

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Camper, D., R. Brigmon and F. Stutzenberger. Bioremediation of compounds hazardous to health and the environment. An overview. In: *Biotransformations: Bioremediation Technology for Health and Environmental Protection.* V.P. Singh (ed.) Elsevier Science Publishers, Netherlands

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EDUCATION

1989	Ph.D., University of Pennsylvania, Bioengineering (Biomaterials)
1986	M.S., University of Pennsylvania, Bioengineering (Biomaterials)
1979	B.S., University of Virginia, Chemical Engineering

PROFESSIONAL EXPERIENCE

1997 – 1998	Visiting Professor, Dept. of Materials, Imperial College, University of London, London, UK
1995 - present	Clemson University, Associate Professor of Bioengineering and Materials Science and Engineering Program
1990 -present	Shriners Hospitals for Crippled Children, Scientific Staff
1989 - 1995:	Clemson University, Assistant Professor of Bioengineering and Materials Science and Engineering Program
1984-1989:	University of Pennsylvania, Research Assistant, Dept. of Bioengineering
1979-1984:	Amoco Oil Co., Oil Refinery Operations and Inspection Engineer

HONORS AND AWARDS

Conference Best Student Paper Award, Zhang G., Latour R.A. Jr. (advisor), FRP Composite Compressive Strength and Its Dependence Upon Interfacial Bond Strength, Fiber Misalignment, & Matrix Nonlinearity, *American Society for Composites*, 8th Technical Conference, Cleveland, OH, 1993.

Recipient of MOSES Award for outstanding service in the development of test standards, F04 Medical Devices Committee, American Society for Testing and Materials, awarded May 1997.

Nominated for Who's Who in Polymers and Plastics, 1999.

PROFESSIONAL ACTIVITIES / MEMBERSHIPS

Review panel member, NSF Integrative Graduate Education and Research Training Program (IGERT), 6/99.

Society for Biomaterials: Chair, Biomolecular Engineering Special Interest Group
Member, Society for Biomaterials (1988-)
Member, American Society for Testing and Materials (1990-)
    F-04 Committee on Medical and Surgical Materials and Devices
    D-30 Committee on High Modulus Fibers and their Composites
American Society for Composites (1991 - 1997)
Meeting Coordinator, Engineers in Orthopaedics, 1996 Meeting, Atlanta, GA.
Meeting Coordinator, Engineers in Orthopaedics, 1997 Meeting, San Francisco, CA.

GRANTS AND CONTRACTS: Approx. $3.4 million, Whitaker Foundation, Veterans Administration, NSF, NIH, Corporate sources.

NUMBER OF REFEREED PUBLICATIONS (papers & proceedings): Approx. 84

PATENTS: 4 issued, 1 pending

R.A. Latour: Refereed publications during past four years.

Zhang G., Latour R.A. Jr., Kennedy J.M., Schutte H.D. Jr., Friedman R.J., 1996. Long Term Compressive Strength Durability of CF/PEEK Composite in Physiologic Saline, *Biomaterials,* 17:781-789

Deng M., Latour R.A. Jr., Drews M.J., and Shalaby S.W., 1996. Effects of Gamma Irradiation, Irradiation Environment, and Post-Irradiation Aging on Thermal and Tensile Properties of Ultrahigh Molecular Weight Polyethylene Fibers, *J. Applied Polymer Science*, 61:2075-2084.

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PERSONAL INFORMATION

Asian, male, married
Naturalized U.S. Citizen

EDUCATION

1989	Ph.D., The Florida State University, Chemistry
1985	M.S., Zhejiang University, China, Chemical Thermodynamics
1982	B.Eng., Zhejiang Institute of Technology, China, Engineering Chemistry

PROFESSIONAL EXPERIENCE

1999- present	Professor of Chemistry and Materials Science & Engineering
1996-1999	Associate Professor, Clemson University
1992-1996	Assistant Professor, Clemson University
1989-1992	Postdoctoral Research Associate, The University of Texas at Austin

HONORS AND AWARDS

Award for Faculty Achievement in Sciences, Clemson University, 1999
Spectroscopy, Editorial Board "15 Brightest Stars in Analytical Spectroscopy", 1995
Graduate Research Award, Florida State University, 1988

PROFESSIONAL ACTIVITIES/MEMBERSHIPS

NSF Workshop on Materials Chemistry, Morristown, New York, October 15-18, 1998.
Editor, Book: *Supercritical Fluid Technology in Materials Science and Engineering: Synthesis, Properties, and Applications*, to be published by Marcel Dekker, New York in 2000.
Organizer, Symposium on Polymeric Fullerene Materials, ACS National Meeting, Aug. 1999.

Member: American Chemical Society
Inter-American Photochemical Society
American Society for Photobiology
The Electrochemical Society.

RESEARCH INTERESTS

Preparation-characterization-application of novel materials. Supercritical fluid processing of nanomaterials.

Y.-P. Sun: Publications in refereed journals in past four years

Journal Publications:

Sun, Y.-P.; Lawson, G. E.; Bunker, C. E.; Johnson, R. A.; Ma, B.; Farmer, C.; Riggs, J. E.; Kitaygorodskiy, A. **1996.** "Preparation and Characterization of Fullerene-Styrene Copolymers" *Macromolecules*, *29*, 8441.

Ma, B.; Sun, Y.-P. **1996.** "Fluorescence Spectra and Quantum Yields of [60]Fullerene and [70]Fullerene under Different Solvent Conditions. A Quantitative Examination Using a Near-Infrared-Sensitive Emission Spectrometer" *J. Chem. Soc. Perkin Trans. 2*, 2157.

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Bunker, C. E.; Rollins, H. W.; Gord, J. R.; Sun, Y.-P. **1997.** "Efficient Photodimerization Reaction of Anthracene in Supercritical Carbon Dioxide" *J. Org. Chem.*, *62*, 7324.

Sun, Y.-P.; Riggs, J. E. **1997.** "Optical Limiting Properties of [60]Fullerene Derivatives" *Chem. Mater.*, *9*, 1268.

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Sun, Y.-P.; Ma, B.; Bunker, C. E. **1998.** "Photoinduced Intramolecular n- * Electron Transfer in Aminofullerene Derivatives" *J. Phys. Chem. A*, *102*, 7580.

Sun, Y.-P.; Rollins, H. W.; Guduru, R. .**1999.** "Preparations of Nickel, Cobalt, and Iron Nanoparticles through the Rapid Expansion of Supercritical Fluid Solutions (RESS) and Chemical Reduction" *Chem. Mater*, *11*, 7.

Riggs, J. E.; Sun, Y.-P. **1999.** "Optical Limiting Properties of [60]Fullerene and Methano[60]fullerene Derivative in Solution versus in Polymer Matrix. The Role of Bimolecular Processes and a Consistent Nonlinear Absorption Mechanism" *J. Phys. Chem.*, *103*, 485.

Sun, Y.-P.; Riggs, J. E.; Rollins, H. W.; Guduru, R. **1999**, "Strong Optical Limiting of Silver-Containing Nanocrystalline Particles in Stable Suspensions" *J. Phys. Chem. B 103*, 77.

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Lawson, G. E.; Guduru, R.; Kitaygorodskiy, A.; Sun, Y.-P. **1999** "Photoinduced Electron Transfer Reactions of [60]Fullerene with Triethylamine" *J. Org. Chem.*, *64*, 5913.

Sun, Y.-P.; Lawson, G. E.; Huang, W.; Wright, A. D.; Moton, D. K. **1999.** "Preparation and Characterization of Highly Water-Soluble Pendant Fullerene Polymers" *Macromolecules*, *32*, 8747.

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Riggs, J. E.; Walker, D. B.; Carroll, D. L.; Sun, Y.-P. "Optical Limiting Properties of Suspended and Solubilized Carbon Nanotubes" *J. Phys. Chem.*.

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Sun, Y.-P. (Editor): *Supercritical Fluid Technology in Materials Science and Engineering: Synthesis, Properties, and Applications*; to be published by Marcel Dekker (New York) in 2000.

Bunker, C. E.; Rollins, H. W.; Sun, Y.-P. "Molecular Interactions in Supercritical Fluids" in *Supercritical Fluid Technology in Materials Science and Engineering: Synthesis, Properties, and Applications*; Sun, Y.-P., Ed., Marcel Dekker: New York, to be published in 2000.

Sun, Y.-P.; Rollins, H. W. "Rapid Expansion of Supercritical Fluid Solutions (RESS) for the Synthesis and Processing of Nanoscopic Materials" in *Supercritical Fluid Technology in Materials Science and Engineering: Synthesis, Properties, and Applications*; Sun, Y.-P., Ed., Marcel Dekker: New York, to be published in 2000.

Other Scholarly Publications:

Sun, Y.-P.; Riggs, J. E. "Nonlinear Absorption and Optical Limiting Properties of Fullerene Materials" *Proc. SPIE-Int. Soc. Opt. Eng.* **1997**, *3142*, 83.

Sun, Y.-P.; Rollins, H. W.; Simmons, K. J. "Properties of Supercritical Fluids and Effects on Polymerization Reactions and Material Processing" *Polymer Preprints* **1997**, *38*, 448.

Sun, Y.-P.; Ma, B.; Riggs, J. E.; Bunker, C. E. "Linear and Nonlinear Optical Properties of Fullerene Derivatives" in *Fullerenes, Recent Advances in the Chemistry and Physics of Fullerenes and Related Materials, Vol. 4*; Kadish, K. M.; Ruoff, R. S., Eds., The Electrochemical Society Inc., **1997**, p. 95.

Sun, Y.-P.; Lawson, G. E.; Wang, N.; Liu, B.; Moton, D. K.; Dabestani, R. "Preparation and Characterization of Highly Water-Soluble Pendant Fullerene Polymers and Fullerene-Protein Conjugates" in *Fullerenes, Recent Advances in the Chemistry and Physics of Fullerenes and Related Materials, Vol. 4*; Kadish, K. M.; Ruoff, R. S., Eds., The Electrochemical Society Inc., **1997**, p. 645.

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Vita

I. Name: Tzuen-Rong J. Tzeng

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II. A. Academic training:

BS (Food Science), Tunghai University, Taichung, Taiwan (1985)

Ph.D. (Microbiology), Clemson University, Clemson, SC (1998)

B. Employment history:

1999 - Present	Consultant: *Poly-Med, Inc.* Center for Applied Technology Pendleton, SC
1998 - Present	Lecturer: Department of Microbiology and Molecular Medicine, Clemson University.
1997 - Present	Technical Support Provider: Greenville Hospital System/Clemson Univers Cooperative
1995 - 1997	Microbiologist, Safety Coordinator and Technical Support Provid Department of Microbiology, Clemson University.
1994 - 1995	Research Assistant: Department of Microbiology, Clemson University.
1989 – 1994	Teaching Assistant: Department of Microbiology, Clemson University.
1994 - 1994	Consultant: *Environment Remedial Services.* Center for Applied Technology Pendleton, SC
1992 - 1994	Consultant: *Southern BioProduct Technologies, Inc.* Center for Applied Technology Pendleton, SC
1987 - 1988	Research Assistant: Department of Food Science, Tunghai University in corporation w *Taichung Fermentation*, Inc., Taichung, Taiwan.

C. Professional Organization:

American Association for the Advancement of Science

New York Academy of Science

D. Honors and Awards:

Thomas Edison Award, Edison Society, 1994

Lexington Who's Who, 2000

III. Refereed Publication during past four years – NA

In Review (As of May 31, 2000)

Sandra P. Story, Stephen H. Parker, Jonathan D. Kline, Tzuen-Rong J. Tzeng, Ellis L. Kline "Identification of four structu genes and two promoters necessary for utilization of polycyclic aromatic hydrocarbons by *Sphingomonas paucimobilis* str EPA505" *Gene*

Dissertation (May 1998)

Degradation of Phenanthrene by *Pseudomonas cepacia* and Localization of Phenenthrene Degradati Structural Gene(s)

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EDUCATION:

Ph.D. Aquatic Toxicology/Biology, Virginia Polytechnic Institute and State University, 1977.
M.S. Botany/Plant Ecology, Clemson University, 1974.
B.S. Botany, Clemson University, 1972.

SELECTED HONORS AND MEMBERSHIP:

Chair, Expert Advisory Committee, Canadian Network of Toxicology Centres, Environment Canada and Health and Welfare, 1996-1999.

Member, Expert Advisory Committee, Canadian Network of Toxicology Centres, Environment Canada and Health and Welfare, 1992-present.

Board of Directors, Society of Environmental Toxicology and Chemistry, 1989-1991; 1995-present. Executive Committee 1997-present; Vice President 1998-1999; President 1999-2000.

Distinguished Southerner, Editors of Southern Living, Water Watchdogs, April 1994.

Publications in refereed journals during past four years

Gillespie, W.B., Jr., W.B. Hawkins, J.H. Rodgers, Jr., M.L. Cano and P.B. Dorn. 2000. Transfers and transformations of zinc in constructed wetlands: Mitigation of a refinery effluent. Ecological Engineering 14: 279-292.

Goodfellow, W. L., L.W. Ansley, D.T. Burton, D.L. Denton, P.B. Dorn, D.R. Grothe, M.A. Heber, T.J. Norberg-King and J.H. Rodgers, Jr. 2000. Major ion toxicity in effluents: A review with permitting recommendations, Environmental Toxicology and Chemistry 19(1): 175-182.

Gillespie, W.B., Jr., W.B. Hawkins, J.H. Rodgers, Jr., M.L. Cano and P.B. Dorn. 1999. Transfers and transformations of zinc in flow-through wetland microcosms. Ecotoxicology and Environmental Safety 43: 126-132.

Huggett, D.B., W.B. Gillespie, Jr. and J.H. Rodgers, Jr. 1999. Copper bioavailability in Steilacoom Lake sediments. Archives of Environmental Contamination and Toxicology 36: 120-123.

Lizotte, R.W., Jr., D.C.L. Wong, P.B. Dorn and J.H. Rodgers, Jr. 1999. Effects of a homologous series of linear alcohol ethoxylate surfactants on fathead minnow early life stages. Archives of Environmental Contamination and Toxicology 37: 536-541.

Moore, M.T., D.B. Huggett, G.M. Huddleston III, J.H. Rodgers, Jr. and C.M. Cooper. 1999. Herbicide effects on Typha latifolia (Linneaus) germination and root and shoot development. Chemosphere 38(15): 3637-3647.

Rodgers, J.H., Jr. 1999. Editorial – Opportunities and challenges for SETAC in the next century. Environmental Toxicology and Chemistry 18(11): 2401-2402.

Solomon, K., D. Bright, P.Hodson, K.-J. Lehtinen, B. McKague and J. Rodgers. 1999. Evaluation of ecological risks associated with the use of chlorine dioxide for the bleaching of pulp. Report prepared for the Alliance for Environmental Technology. 86pp.

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Moore, M.T., D.B. Huggett, W.B. Huggett, W.B.Gillespie, Jr., J.H. Rodgers, Jr., and C.M. Cooper. 1998. Comparative toxicity of chlordane, chlorpyrifos, and aldicarb to four aquatic testing organisms. Archives of Environmental Contamination and Toxicology 34: 152-157.

Chapman, P.M., B. Anderson, S. Carr, V. Engles, R. Green, J. Hameedi, M. Harmon, P. Haverland, J. Hyland, C. Ingersoll, E. Long, J. Rodgers, Jr., M. Salazar, P.K. Sibley, P.J. Smith, R.C. Swartz, B. Thompson and H. Windom. 1997. General giudelines for using the Sediment Quality Triad. Marine Pollution Bulletin 34 (6): 368-372.

Dorn, P.B., Rodgers, Jr., J.H., Gillespie, Jr. W.B., Lizotte, Jr. R.E. and Dunn, A.W., 1997. The Effects of a C12-13 Linear Alcohol Ethoxylate Surfactant on Periphyton Macrophytes, Invertebrates, and Fish in Stream Mesocosms. Environmental Toxicology and Chemistry 16(8): 1634-1645.

Harrelson, R.A., J.H. Rodgers, Jr., R.E. Lizotte, Jr. and P.B. Dorn. 1997. Responses of fish exposed to a C9-11 linear alcohol ethoxylate nonionic surfactant in stream mesocosms. Ecotoxicology 6:321-333.

Hawkins, W.B., Rodgers, Jr. J.H., Gillespie, Jr. W. B. Dunn, A.W., Dorn, P.B., and Cano, M.L. 1997. Design and Construction of Wetlands for Aqueous Transfers and Transformations of Selected Metals. Ecotoxicology and Environmental Safety 36: 238-248.

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Gillespie, Jr., W. B., Rodgers, Jr., J.H. and Crossland, N.O., 1996. Effects of a Nonionic Surfactant (C14-15 AE-7) on Aquatic Invertebrates in Outdoor Stream Mesocosms. Environmental Toxicology and Chemistry 15(8) 1412-1422.

Kline, E.R., Figueroa, R.A., Rodgers, Jr., J.H. and Dorn, P.B. 1996. Effects of a Nonionic Surfactant (C14-15 AE-7) on Fish Survival, Growth and Reproduction in the Laboratory and in Outdoor Stream Mesocosms. Environmental Toxicology and Chemistry 15 (6): 997-1002.

Rodgers, Jr. J.H., Crossland, N.O., Kline, E.R., Gillespie, Jr., Figueroa, R.A., and Dorn, P.B. 1996. Design and Construction of Model Stream Ecosystems. Ecotoxicology and Environmental Safety 33: 30-37.

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Education

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BS	Wildlife Biology	NC State University Raleigh, NC	1980
BS	Poultry Science	NC State University Raleigh, NC	1981
MS	Nutrition	NC State University Raleigh, NC	1987
PhD	Nutrition	NC State University Raleigh, NC	1989

Publications in refereed journals during past four years

1. Fairchild, A. S., J. L. Grimes, M. J. Wineland, and F. T. Jones, 1998. Disk diffusion antimicrobial susceptibility tests against avian *Escherichia coli* isolates. Poultry Sci. 77 (Supp. 1):94.

2. Fairchild, A.S., J.L. Grimes, F.W. Edens, M.J. Wineland, F.T. Jones, and A.E. Sefton. 1999. Effect of hen age, Bio-Mos and Flavomycin on susceptibility of turkey poults to oral Escherichia coli challenge. In: Under the Microscope: Focal Points for the New Millenium. Biotechnology in the Feed Inductry. Proceedings of Alltech's 15th Annual Symposium (T.P. Lyons and K.A. Jacques, eds). Nottingham University Press, Nottingham, UK. pp. 185-201.

3. Crouch, A. N., J. L. Grimes, V. L. Christensen, and J. D. Garlich, 1999. Restriction of feed consumption and body weight in two strains of Large White turkey breeder hens. Poultry Sci. 78:1102-1109.

4. Caldwell, S.R., Johnson, A.F., Yule, T.D., Grimes, J.L., Ficken, M., and V. L. Christensen, 1999. Increased egg production in juvenile turkey hens after active immunization with vasoactive intestinal peptide. Poultry Sci. 78:899-901.

5. Grimes, J.L., and T.D. Siopes, 1999. A survey and overview of lighting practices in the U.S. turkey breeder industry. J. Appl. Poultry Res. 8:493-498.

6. Williams, C.M., J.L. Grimes, and R.L. Mikkelsen, 1999. The use of poultry litter as co-substrate and source of inorganic nutrients and microorganisms for the *Ex Situ* biodegradation of petroleum compounds. Poultry Sci. 78:956-964.

7. Bottcher, R.W., G.R. Baughman, R.D. Munilla, J.L. Grimes, and E. Gonder, 1998. Development of a large paddle fan for cooling poultry. Applied Engineering in Agriculture, Vol. 14(1):87-96.

8. Grimes, J.L., D.V. Maurice, S.F. Lightsey, and J.G. Lopez, 1997. The effect of dietary Femacto on layer hen performance. J. Appl. Poultry Res. 6:399-403.

9. Grimes, J.L., V.L. Christensen, and R. Whittaker, 1997. Filtering and the viability of turkey spermatozoa. J. Appl. Poultry Res. 6:34-38.

10. Crouch, A.N., J.L. Grimes, P.R. Ferket, L.N. Thomas, and A.E. Sefton, 1997. Effect of enzyme supplementation of starter diets for broilers and turkeys to enhance wheat utilization. J. Appl. Poultry Res. 6:147-154.

11. Grimes, J.L., and S.L. Pardue, 1996. A survey of commercial turkey hatcheries in the United States. J. Appl. Poultry Res. 5:231-238.

COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE
NATIONAL RESEARCH INITIATIVE COMPETITIVE GRANTS PROGRAM

Conflict of Interest List

Name: Robert A. Latour

For each investigator (and other personnel as described in the application kit or program description), list ***ALPHABETICALLY*** the full names of **only** the individuals in the following categories. Additional pages may be used as necessary. ***A conflict of interest list for each investigator must be submitted before a proposal is considered complete. Inclusion of a C.V. or publication list in the proposal is not sufficient.***

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Other investigators working in the applicant's specific research area are not in conflict of interest with the applicant unless those investigators fall within one of the listed categories.

CO-AUTHORS

An, Huey H.
Biggers, Sherrill B. Jr.
Boland, Thomas
Chen, Wei
De Andrade, J. Robin
Deng, Meng
Draughn, Robert A.
Drews, Michael J.
Friedman, Richard J.
Fu, Wei
Hench, Larry L.
Johnson, Russell A.
Kennedy, John M.
Lickfield, Gary C.
Ogale, Amod
Powers, Dennis L.
Rinehart, Jason D.
Schutte, Harold D.
Shalaby, Shalaby W.
Srinivasan, Sundar
Stutzenberger, Fred J.

CO-AUTHORS

Taylor, Theodore D.
Tian, Yuan
Trembley, Sharon D.
West, John K.
Wheeler, A. P.
Zhang, Guigen

COLLABORATORS

An, Y. H. H.
Bannar, S.
Biggers, S. B.
Chen, W.
Creager, S. E.
Draughn, R. A.
Friedman, R. J.
Hench, L. L.
Hirt, D. E.
Husson, S. M.
Kennedy, J. K.
Lickfield, G.
Ogale, A. A.
Schutte, H. D.
Shalaby, S. W.
Stuart, S. J.
Stutzenberger, F.
Sun, Y. –P.
Tian, Y.
Von Recum, A. F.
Wagner, K. B.

ADVISEES/ADVISORS

Black, Jonathan
Burke, Elizabeth
Trembley, Sharon
Rinehart, Jason
Srinivasan, Sundar
Foy, Keith
Gross, James
Brattain, Michael
James, Tiffany
Fu, Wei
Cannell, Peter
Granger, Christopher

OTHER – SPECIFY NATURE

Conflict of Interest List

Name: Robert A. Latour -- Continued

For each investigator (and other personnel as described in the application kit or program description), list *ALPHABETICALLY* the full names of **only** the individuals in the following categories. Additional pages may be used as necessary. ***A conflict of interest list for each investigator must be submitted before a proposal is considered complete. Inclusion of a C.V. or publication list in the proposal is not sufficient.***

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Other investigators working in the applicant's specific research area are not in conflict of interest with the applicant unless those investigators fall within one of the listed categories.

CO-AUTHORS	CO-AUTHORS	COLLABORATORS	ADVISEES/ADVISORS
		West, J. K.	Holloway, Clay
		Wheeler, A. P.	Rini, Christopher
			Li, Chaodi
			Vernakar, Varadraj
			Grahek, Garrett
			OTHER - SPECIFY NATURE

COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE
NATIONAL RESEARCH INITIATIVE COMPETITIVE GRANTS PROGRAM

Expires 5 0

Conflict of Interest List

Name: _FRED J. STUTZENBERGER_______________________

For each investigator (and other personnel as described in the application kit or program description), list ***ALPHABETICALLY*** the full names of **only** the individuals in the following categories. Additional pages may be used as necessary. ***A conflict of interest list for each investigator must be submitted before a proposal is considered complete. Inclusion of a C.V. or publication list in the proposal is not sufficient.***

☐ All *co-authors* on publications within the past four years, including pending publications and submissions

☐ All *collaborators* on research projects within the past four years, including current and planned collaborations

☐ All thesis or postdoctoral *advisees/advisors* within the past four years

☐ All persons in your field with whom you have had a *consulting/financial arrangement/other conflict-of-interest* within the past four years

Other investigators working in the applicant's specific research area are not in conflict of interest with the applicant unless those investigators fall within one of the listed categories.

CO-AUTHORS	CO-AUTHORS	COLLABORATORS	ADVISEES/ADVISORS
BODINE, ASHLEY	HAMMON, PAUL	ADLER, PETER,	BURKHARDT, BRANT
BRIGMON, ROBIN	HILL, JOSEPH	STERN, NORMAN	NAYDUCH, DANA
BURKHARDT, BRANT	HUANG, LILI	GRIMES, JESSE	LI, SHINENG
BUSH, JOY	HUNTINGTON, KAY	JENKINS, THOMAS	
BUTLER, ROSS	KHANOLKAR, SUPRIYA	KILGORE, MICHAEL	
CAMPER, DWIGHT	LAWSON, JOHN		
CAVE, DAVID R.	LAWSON, BRIAN		
COLLINS, IVY	LEE, CYNTHIA		
ELLIOTT, KIMBERLY	MASUBUCHI, NAOKO		
FARRIS, HAROLD	MONATH, THOMAS		
FESCEMYER, HOWARD	READ, LEANNA		
FULTON, LINDA	RICHARDSON, YOLANDA		
GANGEMI, DAVID	RILEY, MELISSA		
GRUBEL, PETER	STADTLANDER, CHRISTIAN		OTHER - SPECIFY NATURE

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NATIONAL RESEARCH INITIATIVE COMPETITIVE GRANTS PROGRAM

Conflict of Interest List

Name: Ya-Ping Sun

For each investigator (and other personnel as described in the application kit or program description), list ***ALPHABETICALLY*** the full names of **only** the individuals in the following categories. Additional pages may be used as necessary. ***A conflict of interest list for each investigator must be submitted before a proposal is considered complete. Inclusion of a C.V. or publication list in the proposal is not sufficient.***

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CO-AUTHORS

Bunker, C. E.
Carroll, D. L.
Dabestani, R.
DesMarteau, D. D.
Farmer, C.
Fu, K.
Gord, J. R.
Guduru, R.
Guldi, D. M.
Hajduk, A. M.
Huang, W.
Kafafi, Z. H.
Kadish, K. M.
Kamat, P. V.
Kitaygorodskiy, A.
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Liu, B.
Lytle, C.
Ma, B.
Martin, C. W.
Moton, D. K.

CO-AUTHORS

Mullins, J. E.
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Schanze, K. S.
Shafer, G. J.
Shinar, J.
Simmons, K. J.
Tu, M. -H.
Valentin, S.
Vardeny, Z. V.
Wang, N.
Whiteside, T.
Wright, A. D.
Zhang, X. –F.
Zhang, Y. J.

COLLABORATORS

Carroll, David L.
Connell, John
Donovan-Merkert, Bernadette T.
Dabestani, Reza
DesMarteau, Darryl D.
Gord, James R.
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ADVISEES/ADVISORS

Bunker, Christopher E.
Guduru, Radhakishan
Lawson, Glenn E.
Ma, Bin
Rollins, Harry W.

OTHER - SPECIFY NATURE

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NATIONAL RESEARCH INITIATIVE COMPETITIVE GRANTS PROGRAM

Expires [illegible]

Conflict of Interest List

Name: Tzuen-Rong Jeremy Tzeng

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CO-AUTHORS	CO-AUTHORS	COLLABORATORS	ADVISEES/ADVISORS
Ahn, Eugene		Chen, Wen	Beck, Michael T
Jiang, Huabei		Hayasaka, Steve S	Kline, Ellis L
Kline, Jonathan D		Moore, Mary C	
Parker, Stephen H		Shalaby, Shalaby	
Story, Sandra P			
			OTHER - SPECIFY NATURE

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COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE
NATIONAL RESEARCH INITIATIVE COMPETITIVE GRANTS PROGRAM

Expires 5.00

Conflict of Interest List

Name: John H. Rodgers, Jr.

For each investigator (and other personnel as described in the application kit or program description), list *ALPHABETICALLY* the full names of **only** the individuals in the following categories. Additional pages may be used as necessary. ***A conflict of interest list for each investigator must be submitted before a proposal is considered complete. Inclusion of a C.V. or publication list in the proposal is not sufficient.***

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CO-AUTHORS	CO-AUTHORS	COLLABORATORS	ADVISEES/ADVISORS
Anderson, B.	Haverland, R.A.	Benson, William H.	Bohannon, Amy Leigh
Ansley, L.W.	Hawkins, W.B.	Deardorff, Thomas	Deaver, Emily
Bright, D.	Heber, M.A.	Dorn, Phil, Sr.	Figueroa, Rocio A.
Burton, D.T.	Hodson, P.	Elzerman, Alan W.	Gillespie, Barry
Cano, M.L.	Huddleston, G. M., III	Gladden, John	Hawkins, Bradley
Carr, S.	Hugget, D. B.	Lehman, Roger W.	Heatley, John E.
Chapman, P.M.	Hugget, W. B.	Moore, Matt T.	Huddleston, G. Matt
Cooper, C.M.	Hyland, J.	Myers, Jim	Lizotte, Richard
Crossland, N.O.	Ingersoll, C.	Schmidt, Jim	Masten, Bryan
Deaver, E.	Lehtinen, K.-J.	Scott, Geoffrey	Muller, Stacy L.
Denton, D.L.	Lizotte, R.E., Jr.	Wigley, T. Bently	Murray-Gulde, Cindy
Dorn, P.B.	Lizotte, R. W., Jr.	Wing, Rod A.	Schwartman, Adam L.
Dunn, A.W.	Long, E.		OTHER - SPECIFY NATURE
Engles, V.	McKague, B.		
Gillespie, W. B., Jr.	Moore, M.T.		
Goodfellow, W. L.	Norbert, T.J.		
Green, R.	Rogers, P.L.		
Grothe, D.R.	Salazar, M.		
Hameedi, J.	Sibley, P.K.		
Harmon, M.	Smith, P.J.		
Harrelson, R. A.	Solomon, K.		

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COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE
NATIONAL RESEARCH INITIATIVE COMPETITIVE GRANTS PROGRAM

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Conflict of Interest List

Name: John H. Rodgers, Jr. -- Continued

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CO-AUTHORS	CO-AUTHORS	COLLABORATORS	ADVISEES/ADVISORS
	Suedel, B.C.		Sherrard, Rick M.
	Swartz, R.C.		Winfield, Linda
	Thompson, B.		
	Windom, H.		
	Wong, D.C.L.		
			OTHER - SPECIFY NATURE

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COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE
NATIONAL RESEARCH INITIATIVE COMPETITIVE GRANTS PROGRAM

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Conflict of Interest List

Name: Jesse L. Grimes

For each investigator (and other personnel as described in the application kit or program description), list ***ALPHABETICALLY*** the full names of **only** the individuals in the following categories. Additional pages may be used as necessary. ***A conflict of interest list for each investigator must be submitted before a proposal is considered complete. Inclusion of a C.V. or publication list in the proposal is not sufficient.***

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CO-AUTHORS	CO-AUTHORS	COLLABORATORS	ADVISEES/ADVISORS
Tom Siopes	J. D. Garlich	Mike Williams	J. L. Godwin
Vern Christensen	R. W. Bottcher	Vern Christensen	C. Parks
Frank Edens	G. R. Baughman	Thomas Carter	A. N. Crouch
Andy Crouch	R. D. Munilla	K. E. Anderson	A. S. Fairchild
Mike Williams	E. Gonder	W. E. Donaldson	Mary Fosnaught
Frank Jones	D. V. Maurice	M. J. Wineland	B. Fairchild
A. E. Sefton	S. F. Lightsey	B. W. Sheldon	
A.S. Fairchild	J. G. Lopez		
M. J. Wineland	T. G. Gaylord		
P. R. Ferket	S. R. Caldwell		
L. N. Thomas	A. F. Johnson		OTHER - SPECIFY NATURE
S. L. Pardue	T. D. Yule		
M. Ficken	R. L. Mikkelsen		
B. Fairchild	L. Bagley		

Form CSREES-1233 (11/96)

V. SUBCONTRACTUAL ARRANGMENTS

V.1. Justification for Subcontract

Clemson University serves as the land grant institution of South Carolina and consequently has a large agricultural component in its mission of education, research and extension service to the public. Therefore, the proposed research is highly appropriate to Clemson's land grant tradition. The Department of Microbiology and Molecular Medicine is positioned in the College of Agriculture, Forestry and Life Sciences and has the only complete microbiology program (BS, MS, PhD) in the state. Dr. Stutzenberger (Co-P.I.) has had experience in working with an enteric pathogen (*Helicobacter pylori*) in animal models (mice and squirrel monkeys); nine publications in international refereed journals have been generated from his studies in the last five years. Recently, his research interest has broadened to include *Aeromonas spp.* and the epidemiological role of houseflies as a vector in their dissemination. This experience will be a valuable asset to the program and will be additionally complemented by the efforts of Dr. Tzeng, also of the Department of Microbiology.

V.2. Statement of Work

See Project Description, Section I.3.iv.
MATERIALS & METHODS: PART 3. IN VIVO BIOACTIVITY STUDIES

V.3. Letter of Collaboration: See following page.

North Carolina Cooperative Extension Servic

NC STATE UNIVERSITY

College of Agriculture and Life Sciences
Extension Poultry Science
Scott Hall / Campus Box 7608
Raleigh, North Carolina 27695

919.515.2621 (phone)
919.515.7070 (fax)

June 2, 2000

Dr. Robert A. Latour, Jr.
Bioengineering & Materials Science Engineering
501 Rhodes Research Center
Clemson University
Clemson, SC 29634

Dear Dr. Latour:

Re: Proposal entitled: Adhesin-Specific Nanoparticles for Removal of *Campylobacter jejuni* from Poultry

North Carolina State University is pleased to submit a proposal and budget and to indicate our institution's willingness to participate with Clemson University on the above referenced USDA/CSREES research project. The portion of the project to be conducted at this institution will be under the direction of Drs. Jesse L. Grimes, Brian Sheldon, and Mike Wineland in the Department of Poultry Science and is entitled "Effect of a Novel Polymer on Control Of *Campylobacter* in Poultry".

The North Carolina State University Office of Sponsored Programs administers all grants for the faculty of NC State University. Therefore, any contract negotiations or awards should be with the Office of Sponsored Programs and directed to the attention of Dr. Russ Lea Associate Vice Chancellor for Research Outreach and Extension. Requests for additional information and questions of contractual and administrative concern should be directed to Dr. Lea who can be contacted directly by phone at 919 515-7200, by Fax 919 515-7721, or by e-mail: Russ_Lea@ncsu.edu. Should additional technical information be needed, contact Dr. Grimes by phone at 919 515-5406, by Fax at 919 515-7070, or by e-mail at: Jesse_Grimes@ncsu.edu.

We look forward to a productive collaboration in this program.

Respectfully,

Jesse L Grimes
Dr. Jesse L. Grimes
Associate Professor

Brian Sheldon
Dr. Brian Sheldon
Professor

Michael J Wineland
Dr. Mike Wineland
Professor

Johnny Wynne
Dr. Johnny Wynne
Associate Dean and Director
North Carolina Agricultural Research Service

cc: Dr. G. B. Havenstein, Head, NCSU Poultry Science

Employment and program opportunities are offered to all people regardless of race, color, national origin, sex, age or handicap. North Carolina State University, North Carolina A&T State University, U.S. Department of Agriculture, and local governments cooperating.

UNITED STATES DEPARTMENT OF AGRICULTURE OMB Approved 0524-0022
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE
Expires 7/31/01

BUDGET

Year 1 Budget

ORGANIZATION AND ADDRESS				**USDA AWARD NO.**	
Department of Poultry Science, North Carolina State University, Box 7608, Raleigh, NC 27695-7608				Duration Proposed Months: 6	Duration Awarded Months: ____
PRINICPAL INVESTIGATOR(S)/PROJECT DIRECTORS J.L. Grimes, B.W. Sheldon, and M.J. Wineland				**FUNDS REQUESTED BY PROPOSER**	**FUNDS APPROVED BY CSREES** (If Different)
A. Salaries and Wages	**CSREES FUNDED WORK MONTHS**				
1. No. of Senior Personnel	Calendar	Academic	Summer		
a. ___ (Co)-PI(s)/PD(s)					
b. ___ Senior Associates					
2. No. of Other Personnel (Non-Faculty)					
a. ___ Research Associates-Postdoctorate					
b. ___ Other Professionals					
c. Graduate Students (stipend for 2.5 years)				$ [redacted]	
d. Prebaccalaureate Students				[redacted]	
e. Secretarial-Clerical					
f. Technical, Shop and Other					
Total Salaries and Wages ____				[redacted]	
B. Fringe Benefits (If charged as Direct Costs) (fringe and health insurance)				[redacted]	
C. Total Salaries, Wages, and Fringe Benefits (A plus B) ____				[redacted]	
G. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item).					
E. Materials and Supplies				[redacted]	
F. Travel 1. Domestic (Including Canada)				[redacted]	
2. Foreign (List destination and amount for each trip.)					
G. Publication Costs/Page Charges					
H. Computer (ADPE) Costs					
IV. All Other Direct Costs (Attach supporting data. List items and dollar amounts. Details of subcontracts, including work statements and budget, should be explained in full in proposal.)				[redacted]	
J. Total Direct Costs (C through I) ____				[redacted]	
N. Indirect Costs If Applicable (Specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.) (19%)				[redacted]	
L. Total Direct and Indirect Costs (J plus K) ____				$ [redacted]	
M. Other ____					
N. Total Amount of This Request ____					
O. Cost Sharing (If Required Provide Details)					

Note: Signatures required only for Revised Budget This is Revision No. ____

NAME AND TITLE (Type or print)	**SIGNATURE**	**DATE**
Principal Investigator/Project Director		
Authorized Organizational Representative		

Form CSREES-55 (6/95)

Form CSREES-55 (6/95)
UNITED STATES DEPARTMENT OF AGRICULTURE   OMB Approved 0524-0022
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

BUDGET

Expires 7/31/01

Year 2 Budget

ORGANIZATION AND ADDRESS				**USDA AWARD NO.**	
Department of Poultry Science, North Carolina State University, Box 7608, Raleigh, NC 27695-7608				Duration Proposed Months: 12	Duration Awarded Months:
PRINICPAL INVESTIGATOR(S)/PROJECT DIRECTORS J.L. Grimes, B.W. Sheldon, and M.J. Wineland				**FUNDS REQUESTED BY PROPOSER**	**FUNDS APPROVED BY CSREES** (If Different)
A. Salaries and Wages	**CSREES FUNDED WORK MONTHS**				
1. No. of Senior Personnel	Calendar	Academic	Summer		
a. ___ (Co)-PI(s)/PD(s)					
b. ___ Senior Associates					
2. No. of Other Personnel (Non-Faculty)					
a. ___ Research Associates-Postdoctorate					
b. ___ Other Professionals					
c. Graduate Students (stipend for 2.5 years)				$ [redacted]	
d. Prebaccalaureate Students				[redacted]	
e. Secretarial-Clerical					
f. Technical, Shop and Other					
Total Salaries and Wages ___				[redacted]	
B. Fringe Benefits (If charged as Direct Costs) (fringe and health insurance)				[redacted]	
C. Total Salaries, Wages, and Fringe Benefits (A plus B) ___				[redacted]	
E. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item).					
E. Materials and Supplies				[redacted]	
F. Travel 1. Domestic (Including Canada)				[redacted]	
2. Foreign (List destination and amount for each trip.)					
G. Publication Costs/Page Charges					
H. Computer (ADPE) Costs					
II. All Other Direct Costs (Attach supporting data. List items and dollar amounts. Details of subcontracts, including work statements and budget, should be explained in full in proposal.)				[redacted]	
J. Total Direct Costs (C through I) ___				[redacted]	
L. **Indirect Costs If Applicable** (Specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.) (19%)				[redacted]	
L. **Total Direct and Indirect Costs** (J plus K) ___				$ [redacted]	
M. Other ___					
N. Total Amount of This Request ___					
O. Cost Sharing (If Required Provide Details)					

Note: Signatures required only for Revised Budget   This is Revision No. ___

NAME AND TITLE (Type or print)	**SIGNATURE**	**DATE**
Principal Investigator/Project Director		
Authorized Organizational Representative		

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BUDGET

Year 3 Budget

ORGANIZATION AND ADDRESS				**USDA AWARD NO.**	
Department of Poultry Science, North Carolina State University, Box 7608, Raleigh, NC 27695-7608				Duration Proposed Months: 12	Duration Awarded Months:
PRINICPAL INVESTIGATOR(S)/PROJECT DIRECTORS J.L. Grimes, B.W. Sheldon, and M.J. Wineland				**FUNDS REQUESTED BY PROPOSER**	**FUNDS APPROVED BY CSREES** (If Different)
A. Salaries and Wages	**CSREES FUNDED WORK MONTHS**				
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b. ___ Senior Associates					
2. No. of Other Personnel (Non-Faculty)					
a. ___ Research Associates-Postdoctorate					
b. ___ Other Professionals					
c. Graduate Students (stipend for 2.5 years)				$ [redacted]	
d. Prebaccalaureate Students				[redacted]	
e. Secretarial-Clerical					
f. Technical, Shop and Other					
Total Salaries and Wages ___				[redacted]	
B. Fringe Benefits (If charged as Direct Costs) (tuition, fringe, and health insurance)				[redacted]	
C. Total Salaries, Wages, and Fringe Benefits (A plus B) ___				[redacted]	
F. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item).					
E. Materials and Supplies				[redacted]	
F. Travel 1. Domestic (Including Canada)				[redacted]	
2. Foreign (List destination and amount for each trip.)					
G. Publication Costs/Page Charges				[redacted]	
H. Computer (ADPE) Costs					
III. All Other Direct Costs (Attach supporting data. List items and dollar amounts. Details of subcontracts, including work statements and budget, should be explained in full in proposal.)				[redacted]	
J. Total Direct Costs (C through I) ___				[redacted]	
M. Indirect Costs If Applicable (Specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.) (19%)				[redacted]	
L. Total Direct and Indirect Costs (J plus K) ___				$ [redacted]	
M. Other ___					
N. Total Amount of This Request ___					
O. Cost Sharing (If Required Provide Details)				[redacted]	

Note: Signatures required only for Revised Budget — This is Revision No. ___

NAME AND TITLE (Type or print)	**SIGNATURE**	**DATE**
Principal Investigator/Project Director		
Authorized Organizational Representative		

Form CSREES-55 (6/95)

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB Approved 0524-0022
Expires 7/31/01

BUDGET

Cumulative Budget

ORGANIZATION AND ADDRESS	**USDA AWARD NO.**	
Department of Poultry Science, North Carolina State University, Box 7608, Raleigh, NC 27695-7608	Duration Proposed Months: 30	Duration Awarded Months:
PRINICPAL INVESTIGATOR(S)/PROJECT DIRECTORS J.L. Grimes, B.W. Sheldon, and M.J. Wineland	**FUNDS REQUESTED BY PROPOSER**	**FUNDS APPROVED BY CSREES** (If Different)

	CSREES FUNDED WORK MONTHS				
A. Salaries and Wages	Calendar	Academic	Summer		
1. No. of Senior Personnel					
a. ___ (Co)-PI(s)/PD(s)					
b. ___ Senior Associates					
2. No. of Other Personnel (Non-Faculty)					
a. ___ Research Associates-Postdoctorate					
b. ___ Other Professionals					
c. Graduate Students (stipend for 2.5 years)				$[redacted]	
d. Prebaccalaureate Students				[redacted]	
e. Secretarial-Clerical					
f. Technical, Shop and Other					
Total Salaries and Wages ____				[redacted]	
B. Fringe Benefits (If charged as Direct Costs) (fringe and health insurance)				[redacted]	
C. Total Salaries, Wages, and Fringe Benefits (A plus B) ____				[redacted]	
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item).					
E. Materials and Supplies				[redacted]	
F. Travel 1. Domestic (Including Canada)				[redacted]	
2. Foreign (List destination and amount for each trip.)					
G. Publication Costs/Page Charges				[redacted]	
H. Computer (ADPE) Costs					
I. All Other Direct Costs (Attach supporting data. List items and dollar amounts. Details of subcontracts, including work statements and budget, should be explained in full in proposal.)				[redacted]	
J. Total Direct Costs (C through I) ____				[redacted]	
K. Indirect Costs If Applicable (Specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.) (19%)				[redacted]	
L. Total Direct and Indirect Costs (J plus K) ____				$ [redacted]	
M. Other ____					
N. Total Amount of This Request ____					
O. Cost Sharing (If Required Provide Details)					

Note: Signatures required only for Revised Budget — This is Revision No. ____

NAME AND TITLE (Type or print)	**SIGNATURE**	**DATE**
Principal Investigator/Project Director		
Authorized Organizational Representative		

Budget Narrative:

A. A stipend for one Master of Science graduate student is included in the budget. This project is an excellent opportunity to provide training and education for a graduate student. In addition, salary for part time student labor is included. By participating in part time work associated with this project, undergraduate students can mentor with faculty and observe research in action.

Year 1: $ [redacted]for graduate student for ½ year and $[redacted]for undergraduate student(s).
Year 2: $[redacted]for graduate student and $[redacted]for undergraduate student(s).
Year 3: $[redacted]for graduate student and $[redacted]for undergraduate student(s).

Fringe benefits include fringe rates at .0825 % and health insurance for the graduate student.
Year 1: $[redacted] fringe benefits for all student workers and $[redacted]for graduate student health insurance.
Year 2: $[redacted]fringe benefits for all student workers and $[redacted]for graduate student health insurance.
Year 3: $[redacted] fringe benefits for all student workers and $[redacted]for graduate student health insurance.

E. Year 1: $[redacted] for materials and supplies to include costs for birds, feed, bedding material, and laboratory supplies such as agar and glass ware.

Year 2: $[redacted]for materials and supplies to include costs for birds, feed, bedding material, and laboratory supplies such as agar and glass ware.

Year 3: $[redacted] for materials and supplies to include costs for birds, feed, bedding material, and laboratory supplies such as agar and glass ware.

E. Year 1: $[redacted]for travel includes expenses for travel to

A: Clemson University to discuss and plan work ($300 for air fare and $200 for lodging for student and principle investigator) and;

B: to scientific meetings to present research results($300 for air fare and $200 for lodging for 1 graduate student).

Year 2: $[redacted]for travel includes expenses for travel to scientific meetings to present research results($[redacted]for air fare and $[redacted]for lodging for 1 graduate student for two professional meetings) and

Year 3: $[redacted] for travel includes expenses for travel to

A: Clemson University to discuss results and plan dissemination to poultry industry and consumers ($[redacted] for air fare and $[redacted] for lodging for student and principle investigators) and;

B: to scientific meetings to present research results($[redacted] for air fare and $[redacted]for lodging for 1 graduate student for two professional meetings) and

C: travel to discuss results with poultry industry and consumers at extension meetings ($1,000)

G: Publication costs for year 3 include page charges for publication in professional journal and reprints of publications to disseminate to poultry industry and consumers.

I. Other indirect cost listed is tuition for the graduate student at $[redacted] for 1 semester tuition for year 3 and $[redacted]per year for years 2 and 3.

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB Approved 0524-0022
Expires 7/31/01

BUDGET – YEAR 1

ORGANIZATION AND ADDRESS				**USDA AWARD NO.**	
Clemson University 300 Brackett Hall, Clemson, SC 29634-5702				Duration Proposed Months: 12	Duration Awarded Months: ____
PRINICPAL INVESTIGATOR(S)/PROJECT DIRECTORS Robert A. Latour, Fred Stutzenberger, Ya-Ping Sun, John Rodgers, T. R. Tzeng				**FUNDS REQUESTED BY PROPOSER**	**FUNDS APPROVED BY CSREES** (If Different)
A. Salaries and Wages	**CSREES FUNDED WORK MONTHS**				
1. No. of Senior Personnel	Calendar	Academic	Summer		
a. 2 (Co)-PI(s)/PD(s)			2	[redacted]	
b. 3 Senior Associates			2	[redacted]	
2. No. of Other Personnel (Non-Faculty) a. ___ Research Associates-Postdoctorate					
b. ___ Other Professionals					
c. 3 Graduate Students				[redacted]	
d. Prebaccalaureate Students					
e. Secretarial-Clerical					
f. Technical, Shop and Other					
Total Salaries and Wages ____				[redacted]	
B. Fringe Benefits (If charged as Direct Costs)				[redacted]	
C. Total Salaries, Wages, and Fringe Benefits (A plus B) ____				[redacted]	
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item).					
E. Materials and Supplies				[redacted]	
F. Travel 1. Domestic (Including Canada)					
2. Foreign (List destination and amount for each trip.)					
G. Publication Costs/Page Charges					
H. Computer (ADPE) Costs					
I. All Other Direct Costs (Attach supporting data. List items and dollar amounts. Details of subcontracts, including work statements and budget, should be explained in full in proposal.)				[redacted]	
J. Total Direct Costs (C through I) ____				[redacted]	
K. Indirect Costs If Applicable (Specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.) 19% of Total				[redacted]	
L. Total Direct and Indirect Costs (J plus K) ____				[redacted]	
M. Other ____					
N. Total Amount of This Request ____				[redacted]	
O. Cost Sharing (If Required Provide Details)					

Note: Signatures required only for Revised Budget	This is Revision No. ____	
NAME AND TITLE (Type or print)	**SIGNATURE**	**DATE**
Principal Investigator/Project Director Robert A. Latour, PI		
Authorized Organizational Representative Y. T. Shah, Chief Research Officer & Sr. Vice Provost		

Form CSREES-55 (6/95)

UNITED STATES DEPARTMENT OF AGRICULTURE OMB Approved 0524-0022
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE
Expires 7/31/01

BUDGET – YEAR 2

ORGANIZATION AND ADDRESS	**USDA AWARD NO.**	
Clemson University 300 Brackett Hall, Clemson, SC 29634-5702	Duration Proposed Months: 12	Duration Awarded Months: ____
PRINICPAL INVESTIGATOR(S)/PROJECT DIRECTORS Robert A. Latour, Fred Stutzenberger, Ya-Ping Sun, John Rodgers, T. R. Tzeng	**FUNDS REQUESTED BY PROPOSER**	**FUNDS APPROVED BY CSREES** (If Different)

	CSREES FUNDED WORK MONTHS				
A. Salaries and Wages					
1. No. of Senior Personnel	Calendar	Academic	Summer		
a. __2_ (Co)-PI(s)/PD(s)			2	[redacted]	
b. __3_ Senior Associates			3	[redacted]	
2. No. of Other Personnel (Non-Faculty)					
a. ___ Research Associates-Postdoctorate					
b. ___ Other Professionals					
c. 4 Graduate Students				[redacted]	
d. Prebaccalaureate Students					
e. Secretarial-Clerical					
f. Technical, Shop and Other					
Total Salaries and Wages ____				[redacted]	
B. Fringe Benefits (If charged as Direct Costs)				[redacted]	
C. Total Salaries, Wages, and Fringe Benefits (A plus B) ____				[redacted]	
E. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item).					
E. Materials and Supplies				[redacted]	
F. Travel 1. Domestic (Including Canada)				[redacted]	
2. Foreign (List destination and amount for each trip.)					
G. Publication Costs/Page Charges				[redacted]	
H. Computer (ADPE) Costs					
II. All Other Direct Costs (Attach supporting data. List items and dollar amounts. Details of subcontracts, including work statements and budget, should be explained in full in proposal.)				[redacted]	
J. Total Direct Costs (C through I) ____				[redacted]	
L. **Indirect Costs If Applicable** (Specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.)				[redacted]	
L. **Total Direct and Indirect Costs** (J plus K) ____				[redacted]	
M. Other ____					
N. Total Amount of This Request ____				[redacted]	
O. Cost Sharing (If Required Provide Details)				[redacted]	

Note: Signatures required only for Revised Budget

This is Revision No. ____

NAME AND TITLE (Type or print)	SIGNATURE	DATE
Principal Investigator/Project Director Robert A. Latour, PI		
Authorized Organizational Representative Y. T. Shah, Chief Research Officer & Sr. Vice Provost		

Form CSREES-55 (6/95)

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COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

Expires 7/31/01

BUDGET – YEAR 3

ORGANIZATION AND ADDRESS				USDA AWARD NO.	
Clemson University 300 Brackett Hall, Clemson, SC 29634-5702				Duration Proposed Months: 12	Duration Awarded Months: ____
PRINICPAL INVESTIGATOR(S)/PROJECT DIRECTORS Robert A. Latour, Fred Stutzenberger, Ya-Ping Sun, John Rodgers, T. R. Tzeng				**FUNDS REQUESTED BY PROPOSER**	**FUNDS APPROVED BY CSREES** (If Different)
A. Salaries and Wages	**CSREES FUNDED WORK MONTHS**				
1. No. of Senior Personnel	Calendar	Academic	Summer		
a. _2_ (Co)-PI(s)/PD(s)			2	[redacted]	
b. _3_ Senior Associates			3	[redacted]	
2. No. of Other Personnel (Non-Faculty)					
a. ___ Research Associates-Postdoctorate					
b. ___ Other Professionals					
c. 4 Graduate Students				[redacted]	
d. Prebaccalaureate Students					
e. Secretarial-Clerical					
f. Technical, Shop and Other					
Total Salaries and Wages ____				[redacted]	
B. Fringe Benefits (If charged as Direct Costs)				[redacted]	
C. Total Salaries, Wages, and Fringe Benefits (A plus B) ____				[redacted]	
F. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item).					
E. Materials and Supplies				[redacted]	
F. Travel 1. Domestic (Including Canada) 2. Foreign (List destination and amount for each trip.)				[redacted]	
G. Publication Costs/Page Charges				[redacted]	
H. Computer (ADPE) Costs					
III. All Other Direct Costs (Attach supporting data. List items and dollar amounts. Details of subcontracts, including work statements and budget, should be explained in full in proposal.)				[redacted]	
J. Total Direct Costs (C through I) ____				[redacted]	
M. Indirect Costs If Applicable (Specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.)				[redacted]	
L. Total Direct and Indirect Costs (J plus K) ____				[redacted]	
M. Other ____					
N. Total Amount of This Request ____				[redacted]	
O. Cost Sharing (If Required Provide Details)				[redacted]	

Note: Signatures required only for Revised Budget — This is Revision No. ____

NAME AND TITLE (Type or print)	**SIGNATURE**	**DATE**
Principal Investigator/Project Director Robert A. Latour, PI		
Authorized Organizational Representative Y. T. Shah, Chief Research Officer & Sr. Vice Provost		

Form CSREES-55 (6/95)

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COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

BUDGET – CUMULATIVE

Expires 7/31/01

ORGANIZATION AND ADDRESS Clemson University 300 Brackett Hall, Clemson, SC 29634-5702				**USDA AWARD NO.**	
PRINICPAL INVESTIGATOR(S)/PROJECT DIRECTORS Robert A. Latour, Fred Stutzenberger, Ya-Ping Sun, John Rodgers, T. R. Tzeng				Duration Proposed Months: ____36____ **FUNDS REQUESTED BY PROPOSER**	Duration Awarded Months: ________ **FUNDS APPROVED BY CSREES** (If Different)
A. Salaries and Wages	**CSREES FUNDED WORK MONTHS**				
1. No. of Senior Personnel	Calendar	Academic	Summer		
a. __2_ (Co)-PI(s)/PD(s)			6	[redacted]	
b. __3_ Senior Associates			8	[redacted]	
2. No. of Other Personnel (Non-Faculty)					
a. ___ Research Associates-Postdoctorate					
b. ___ Other Professionals					
c. 4 Graduate Students				[redacted]	
d. Prebaccalaureate Students					
e. Secretarial-Clerical					
f. Technical, Shop and Other					
Total Salaries and Wages ____				[redacted]	
B. Fringe Benefits (If charged as Direct Costs)				[redacted]	
C. **Total Salaries, Wages, and Fringe Benefits (A plus B)** ____				[redacted]	
G. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item).					
E. Materials and Supplies				[redacted]	
F. Travel 1. Domestic (Including Canada) 2. Foreign (List destination and amount for each trip.)				[redacted]	
G. Publication Costs/Page Charges				[redacted]	
H. Computer (ADPE) Costs					
IV. All Other Direct Costs (Attach supporting data. List items and dollar amounts. Details of subcontracts, including work statements and budget, should be explained in full in proposal.)					
J. **Total Direct Costs** (C through I) ____				[redacted]	
N. **Indirect Costs If Applicable** (Specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.)				[redacted]	
L. **Total Direct and Indirect Costs** (J plus K)				[redacted]	
M. Other ____				[redacted]	
N. Total Amount of This Request ____					
O. Cost Sharing (If Required Provide Details) ____				[redacted]	

Note: Signatures required only for Revised Budget

This is Revision No. ____

NAME AND TITLE (Type or print)	**SIGNATURE**	**DATE**
Principal Investigator/Project Director Robert A. Latour, PI		
Authorized Organizational Representative Y. T. Shah, Chief Research Officer & Sr. Vice Provost		

Form CSREES-55 (6/95)

BUDGET JUSTIFICATION

Personnel

The proposed integrated project is very multidisciplinary and therefore must involve several faculty and graduate students to cover the entire program.

One month salary support is requested for senior personnel each year to cover time spent planning and conducting the overall program. Dr. Stutzenberger (Co-P.I., College of Forestry, Agriculature, and Life Sciences) and Dr. Latour (Co-P.I., College of Engineering and Science) will supervise and coordinate the each component of the program within their respective college. Dr. Sun does not request any salary support for his portion of the program and Dr. Rodgers salary support will only involve the in vivo exposure sensitivity studies which will not begin until year 2. Dr. Tzeng requests one month salary support in year 1 and two month salary support in years 2 and 3 during which time he will directly supervise the in vitro bioactivity studies.

Funding to cover several graduate students is requested to cover the proposed program. In year 1, two graduate research assistants will be required for nanoparticle synthesis and one will be required to begin preparations for the in vitro bioactivity studies. In years 2 and 3, four graduate research assistants will be required; two for nanoparticle synthesis, one for the in vitro bioactivity studies, and one additional student will begin the in vitro and in vivo exposure sensitivity studies.

Equipment

There are no equipment related costs associated with the proposed program. All equipment necessary for conducting the proposed program is currently in place.

Travel

Travel funds are requested primarily in years 2 and 3 to cover any travel necessary for Clemson personnel to meet with NC State personnel for program related meetings. In addition, travel funding is requested to offset costs involved for faculty and students to attend professional meetings in order to present results from this research program and keep current with research in their professional fields relevant to this program.

Materials and Supplies

Funding to cover materials and supplies are requested in each year as needed to cover each of the four components of this program which will be conducted at Clemson University: nanoparticle synthesis, in vitro bioactivity, in vitro exposure sensitivity, and in vivo exposure sensitivity. $5,000 is required to cover the base polymer, metal oxides, and bioactive molecules for nanoparticle synthesis in addition to chemical and glassware, $3,500 is required each year to cover glassware, reagents, and cell culture expenses for the in vitro bioactivity studies, $1,000 is required in year 2 and 3 for the in vitro exposure sensitivity testing, $32,000 over years 2 and 3 is requested to cover the animal studies for the in vivo exposure testing including histopathology.

Miscellaneous cost funds are requested for general miscellaneous materials and supplies at a level of $1,000 per graduate student, and finally funds are requested to cover reference materials, communication cost for long distance telephone and mail, and publication costs for results presentation.

Subcontract to NC State

In vivo bioactivity assessment studies in poultry will be conducted under this program at NC State University under the direct supervision of Dr. Jesse Grimes. This work will begin at the later half of year one and continue through the end of year 3. The detailed budget and budget justification for this subcontract are presented in Section V, Subcontractual Arrangements.

Statement Regarding Need for Matching Funds

It is requested that CSREES waive the requirement for matching funds for the proposed program because the results of this project will be broadly applicable to any food commodity, not just the poultry industry.

UNITED STATES DEPARTMENT OF AGRICULTURE OMB Approved 0524-0022
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE Expires 7/31/(

CURRENT AND PENDING SUPPORT

Instructions:

1. Record information for active and pending projects. (Concurrent submission of a proposal to other organizations will not prejudice its review by CSREES)
2. All current research to which principal investigator(s) and other senior personnel have committed a portion of their time must be listed, whether or not salary for the person involved is included in the budgets of the various projects.
3. Provide analogous information for all proposed research which is being considered by, or which will be submitted in the near future to, other possible sponsors including other USDA programs.

NAME (List PI #1 first)	SUPPORTING AGENCY AND AGENCY NUMBER	TOTAL $ AMOUNT	EFFECTIVE AND EXPIRATION DATES	% OF TIME COMMITTED	TITLE OF PROJECT
	Current:				
Fred J. Stutzenberger	None				
	Pending:				
Fred J. Stutzenberger	USDA (This proposal)	$[redacted]	01/01/2001 – 12/31/2003	8%	Adhesin-Specific Nanoparticles for Removal of Campylobacter jejuni from Poultry
Fred J. Stutzenberger	USDA	$[redacted]	07/01/2000 – 06/30/2002	11%	Houseflies as a Vector for Foodborne Aeromonads
Fred J. Stutzenberger	Army Research Office	$[redacted]	08/15/2000 – 08/15/2003	11%	A Novel Synthetic Polymer Decontamination Agent for Biological Defense Applications

UNITED STATES DEPARTMENT OF AGRICULTURE OMB Approved
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

Expires 7/31/01

CURRENT AND PENDING SUPPORT

Instructions:

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3. Provide analogous information for all proposed research which is being considered by, or which will be submitted in the near future to, other possible sponsors including other USDA programs.

NAME (List PI #1 first)	SUPPORTING AGENCY AND AGENCY NUMBER	TOTAL $ AMOUNT	EFFECTIVE AND EXPIRATION DATES	% OF TIME COMMITTED	TITLE OF PROJECT
	Current:				
Robert A. Latour	NSF/GA Inst. Of Technology	$[redacted]	08/15/2000 – 08/14/2002	8%	Structural Changes in Fibronectin Binding Domains Resulting from Adsorption to Substrates of Well-Defined Chemistries
Robert A. Latour	NSF/EPSCoR	$[redacted]	01/01/2000 – 12/31/2002	12.5%	Infrastructure for Materials Based Biotechnology
	Pending:				
Robert A. Latour	Army Research Office	$[redacted]	08/15/2000 – 08/14/2003	12.5%	A Novel Synthetic Polymer Decontamination Agent for Biological Defense Applications
Robert A. Latour	NSF	$[redacted]	08/15/2000 – 08/14/2003	12.5%	Quantification of Submolecular Interactions Controlling Protein Adsorption
Robert A. Latour	USDA (This proposal)	$[redacted]	01/01/2001 – 12/31/2003	8%	Adhesin-Specific Nanoparticles for Removal of Campylobacter jejuni from Poultry

UNITED STATES DEPARTMENT OF AGRICULTURE OMB Approved 0524-0022
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE
Expires 7/31/

CURRENT AND PENDING SUPPORT

Instructions:

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2. All current research to which principal investigator(s) and other senior personnel have committed a portion of their time must be listed, whether or not salary for th person involved is included in the budgets of the various projects.
3. Provide analogous information for all proposed research which is being considered by, or which will be submitted in the near future to, other possible sponsor including other USDA programs.

NAME (List PI #1 first)	SUPPORTING AGENCY AND AGENCY NUMBER	TOTAL $ AMOUNT	EFFECTIVE AND EXPIRATION DATES	% OF TIME COMMITTED	TITLE OF PROJECT
	Current:				
Ya-Ping Sun	NSF	[redacted]	1998 – 2001	15%	Experimental Investigations of Fullerene Materials and Polymers. Optical Properties and Nonlinear Absorptive Applications
Ya-Ping Sun	NASA	$[redacted]	2000 – 2001	5%	Nanocomposite Materials for Space Applications
Ya-Ping Sun	NASA	$[redacted]	2000 – 2001	5%	Polymeric/Carbon Nanocomposites for Space Applications
Ya-Ping Sun	NASA	$[redacted]	2000 – 2003	10%	Solubilized Carbon Nanotubes for Space Durable Composite Materials
Ya-Ping Sun	NSF	$[redacted]	1999 – 2001	15%	Nanomaterials with Novel Optical and Electronic Properties
Ya-Ping Sun	NSF/ERC for Advanced Engineering Fibers and Films	$[redacted]	1998 – 2003	15%	Supercritical Solution Processing
Ya-Ping Sun	DoE	$[redacted]	1999 – 2001	10%	Novel Fluorinated Polyelectrolytes for Batteries and Fuel Cells
Ya-Ping Sun	NSF	$[redacted]	1997 – 2000	0%	Acquisition of a High-Field Multi-Nuclear NMR Spectrometer
Ya-Ping Sun	NSF	$[redacted]	1998 – 2001	0%	Acquisition of SQUID Magnetometer
	Pending:				
Ya-Ping Sun	USDA (This proposal)	$[redacted]	01/01/2001 – 12/31/2003	0%	Adhesin-Specific Nanoparticles for Removal of Campylobacter jejuni from Poultry
Ya-Ping Sun	Celanese Corp.	$[redacted]	2000 – 2001	5%	Nanocomposites
Ya-Ping Sun	DoE	$[redacted]	2000 – 2003	8%	Nanomaterials for Energy Related Applications
Ya-Ping Sun	NASA	$[redacted]	2000 – 2003	8%	Development of Polymeric/Carbon Nanocomposite: for Space Applications
Ya-Ping Sun	DoD/ARO	$[redacted]	2000 – 2003	8%	A Novel Synthetic Polymer Decontamination Agent for Biological Defense Applications

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COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE Expires 7/31/

CURRENT AND PENDING SUPPORT

Instructions:

1. Record information for active and pending projects. (Concurrent submission of a proposal to other organizations will not prejudice its review by CSREES)
2. All current research to which principal investigator(s) and other senior personnel have committed a portion of their time must be listed, whether or not salary for th person involved is included in the budgets of the various projects.
3. Provide analogous information for all proposed research which is being considered by, or which will be submitted in the near future to, other possible sponsor including other USDA programs.

NAME (List PI #1 first)	SUPPORTING AGENCY AND AGENCY NUMBER	TOTAL $ AMOUNT	EFFECTIVE AND EXPIRATION DATES	% OF TIME COMMITTED	TITLE OF PROJECT
Tzuen-Rong Tzeng	Current: None				
Tzuen-Rong Tzeng	Pending: USDA (This proposal)	$[redacted]	01/01/2001 – 12/31/2003	17%	Adhesin-Specific Nanoparticles for Removal of Campylobacter jejuni from Poultry

Form CSREES-663 (6/95)

UNITED STATES DEPARTMENT OF AGRICULTURE OMB Approved 0524-0022
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE Expi

CURRENT AND PENDING SUPPORT

Instructions:

1. Record information for active and pending projects. (Concurrent submission of a proposal to other organizations will not prejudice its review by CSREES)
2. All current research to which principal investigator(s) and other senior personnel have committed a portion of their time must be listed, whether or not sal. person involved is included in the budgets of the various projects.
3. Provide analogous information for all proposed research which is being considered by, or which will be submitted in the near future to, other possible including other USDA programs.

NAME (List PI #1 first)	SUPPORTING AGENCY AND AGENCY NUMBER	TOTAL $ AMOUNT	EFFECTIVE AND EXPIRATION DATES	% OF TIME COMMITTED	TITLE OF PROJECT
	Current:				
John H. Rodgers, Jr.	SCUREF/DoE	$[redacted]	1/99 – 2/01	5%	Wetlands for Wastewater Treatment at Sa River Site
John H. Rodgers, Jr.	SCUREF/DoE	$[redacted]	7/99 – 9/01	5%	A-01 Outfall Constructed Wetlands
John H. Rodgers, Jr.	Shell Oil Products	$[redacted]	4/98 – 4/01	1%	Assistance with Design and Construction Wetland for Wastewater Treatment
John H. Rodgers, Jr.	International Paper Company	$[redacted]	8/98 – 12/01	1%	Constructed Wetland for Wastewater Trea IP's Mansfield, LA Facility
John H. Rodgers, Jr.	Applied Biochemists	$[redacted]	1/00 – 1/01	1%	Investigations of Pesticide Toxicity
John H. Rodgers, Jr.	NCASI	$[redacted]	4/00 – 9/00	1%	Review Forest Herbicide Toxicity
	Pending:				
John H. Rodgers, Jr.	USDA (This proposal)	$[redacted]	01/01 - 12/03	8%	Adhesin-Specific Nanoparticles for Remov Campylobacter jejuni from Poultry
John H. Rodgers, Jr.	International Paper Company	$[redacted]	6/00 – 7/01	1%	Design and Construction of a Wetland for Treatment
John H. Rodgers, Jr.	Earth Tec, Inc.	$[redacted]	8/00 – 8/02	1%	Pesticide Toxicity and Fate

Form CSREES-663 (6/95)

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB Approved 0524-0022
Expires 7/31/01

CURRENT AND PENDING SUPPORT

Instructions:

1. Record information for active and pending projects. (Concurrent submission of a proposal to other organizations will not prejudice its review by CSREES)
2. All current research to which principal investigator(s) and other senior personnel have committed a portion of their time must be listed, whether or not salary for the person involved is included in the budgets of the various projects.
3. Provide analogous information for all proposed research which is being considered by, or which will be submitted in the near future to, other possible sponsors including other USDA programs.

NAME (List PI #1 first)	SUPPORTING AGENCY AND AGENCY NUMBER	TOTAL $ AMOUNT	EFFECTIVE AND EXPIRATION DATES	% OF TIME COMMITTED	TITLE OF PROJECT
Jesse L. Grimes	Current:				
	Resource Enhancement Technologies, Inc	$ [redacted]	11/1/99 –12/31/01		Poultry litter reclamation and conversion process project
	BASF Corp., Germany	$ [redacted]	9/98 – 6/00		Effect of enzyme supplementation in wheat-based Diets on nutrient availability
	Alltech, Inc.	$ [redacted]	8/12/99-8/11/01		The effect of organic copper and copper sulfate on Broiler performance, liver copper, and fecal copper
	Alltech, Inc.	$ [redacted]	10/21/98-6/30/00		Use of veg pro for growing broilers
	Advanced Energy Corp.	$ [redacted]	3/22/00-3/21/01		Evaluation of an oxygenated water system (Oxygen) for broilers
	UNC Institute of Nutrition	$ [redacted]	7/1/99-6/30/00		Effect of organic copper on the growth and performance of broiler chickens
	Pending:				
	Alltech, Inc.	$ [redacted]	1/1/00-12/31/01		The effect of dietary phosphorus and enzyme levels on the reproductive performance of turkey breeder hens
	UNC Institute of Nutrition	$ [redacted]	7/1/00-6/30/01		The effect of phytase and low dietary phosphorus on the reproductive performance of Large White turkey breeder hens

Form CSREES-663 (6/95)

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB Approved 0524-0022
Expires 7/31/01

ASSURANCE STATEMENT(S)

STATEMENT OF POLICY - Safeguarding the rights and welfare of subjects at risk and the proper isolation security of research agents in activities supported by Cooperative State Research, Education, and Extension Service is the responsibility of the institution to which support is provided. In order to provide for the adequate discharge of this responsibility, USDA policy requires a formal assurance that appropriate committees in each institution will carry out both initial review of proposals and continuing review of supported projects. The Department also requires certification of such reviews.

NOTE: Check appropriate statements, supplying additional information when necessary

1. INSTITUTION Clemson University	2. TYPE [X] New [ ] Extension [ ] Revision 3. CSREES PROJECT NUMBER OR AWARD NUMBER (If Known)
4. TITLE OF PROJECT Adhesin-Specific Nanoparticles for Removal of *Campylobacter jejuni* in Poultry	5. PRINCIPAL INVESTIGATOR(S) Robert A. Latour Fred J. Stutzenberger

A. RECOMBINANT DNA OR RNA RESEARCH

[X] Project does not involve recombinant DNA or RNA.
[ ] Project involves recombinant DNA or RNA. (Check the applicable statement).
  [ ] This project has been determined by the local IBC to be exempt from the NIH Guidelines.
  [ ] This project is under review by the local IBC and a revised Form CSREES-662 will be submitted when the review is completed.
  [ ] This project has been reviewed by an IBC and was approved on ____________(Date).

This institution agrees to assume primary responsibility for complying with both the intent and procedures of the National Institutes of Health's (NIH) "Guidelines for Research Involving Recombinant DNA Molecules," as revised (see subsection 205(b)(3), Subpart U of the "Uniform Federal Assistance Regulations" (7 CFR Part 3015) and other applicable Federal/State guidelines and regulations.

This responsibility includes:

1. Ensuring that a standing Institutional Biosafety Committee (IBC) reviews proposed projects.
2. Registering with the IBC all experiments involving recombinant DNA and RNA Molecules conducted with the funds provided under this project/grant and complying with the requirements specified in Part II of the NIH Guidelines or any other pertinent guidelines and regulations. IBC's are required to keep records of this research in a form that is available to the U.S. Department of Agriculture (USDA) upon request.

In addition, principal investigators must report the following to the USDA and to their IBC's:

1. New Technical information relating to risks and safety procedures.
2. Serious accidents or releases involving recombinant DNA or RNA.
3. Serious illness of a laboratory worker which may be project related.
4. Other safety problems.

B. ANIMAL CARE

[ ] Project does not involve use of vertebrate animals.
[X] Project involves use of vertebrate animals. (Check the applicable statement).
  [ ] a) The project is in compliance with the Animal Welfare Act of 1966 and 9 CFR Subchapter A (Laboratory Animals), as amended.
  [X] b) This project is under review by the Institutional Care and Use Committee and a revised Form CSREES-662 will be submitted when the review is completed.
  [ ] c) This project has been approved by the Institutional Animal Care and Use Committee on ______________(Date).

C. PROTECTION OF HUMAN SUBJECTS

[X] Project does not involve use of human subjects.
[ ] Project involves use of human subjects. (Check the applicable statement).
  [ ] a) This project includes activities involving human subjects but can in no way be considered at risk. (If this statement is checked, the person signing this form must also initial in the space at right.)______________(Initial)
  [ ] b) This project is under review by an institutional committee as provided by our assurance and a revised Form CSREES-662 will be submitted when the review is completed.
  [ ] c) This project includes activities involving human subjects. Our institutional committee reviewed and approved it on ____________ ___ in accordance with our assurance approved by S&E/and/or DHHS. The project will be subject to continuing review as provided for in that assurance.

SIGNATURE OF AUTHORIZED ORGANIZATIONAL REPRESENTATIVE	TITLE	DATE
Dr. Y. T. Shah YTShah	Chief Research Officer and Senior Vice Provost	6/6/00

Form CSREES-662 (6/95)

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

ASSURANCE STATEMENT(S)

OMB Approved 0524-0022
Expires 7/31/01

STATEMENT OF POLICY - **Safeguarding the rights and welfare of subjects at risk and the proper isolation security of research agents in activities supported by Cooperative State Research, Education, and Extension Service is the responsibility of the institution to which support is provided. In order to provide for the adequate discharge of this responsibility, USDA policy requires a formal assurance that appropriate committees in each institution will carry out both initial review of proposals and continuing review of supported projects. The Department also requires certification of such reviews.**

NOTE: **Check appropriate statements, supplying additional information when necessary**

1. INSTITUTION North Carolina State University	2. TYPE [X] New [ ] Extension [ ] Revision 3. CSREES PROJECT NUMBER OR AWARD NUMBER (If Known)
4. TITLE OF PROJECT Effect of a Novel Polymer on the Control of *Campylobacter* in Poultry	5. PRINCIPAL INVESTIGATOR(S) Jesse L. Grimes, Brian W. Sheldon, Mike J. Wineland

A. RECOMBINANT DNA OR RNA RESEARCH

[x] Project does not involve recombinant DNA or RNA.
[ ] Project involves recombinant DNA or RNA. (Check the applicable statement).
  [ ] This project has been determined by the local IBC to be exempt from the NIH Guidelines.
  [ ] This project is under review by the local IBC and a revised Form CSREES-662 will be submitted when the review is completed.
  [ ] This project has been reviewed by an IBC and was approved on ____________(Date).

This institution agrees to assume primary responsibility for complying with both the intent and procedures of the National Institutes of Health's (NIH) "Guidelines for Research Involving Recombinant DNA Molecules," as revised (see subsection 205(b)(3), Subpart U of the "Uniform Federal Assistance Regulations" (7 CFR Part 3015) and other applicable Federal/State guidelines and regulations.

This responsibility includes:

1. Ensuring that a standing Institutional Biosafety Committee (IBC) reviews proposed projects.
2. Registering with the IBC all experiments involving recombinant DNA and RNA Molecules conducted with the funds provided under this project/grant and complying with the requirements specified in Part II of the NIH Guidelines or any other pertinent guidelines and regulations. IBC's are required to keep records of this research in a form that is available to the U.S. Department of Agriculture (USDA) upon request.

In addition, principal investigators must report the following to the USDA and to their IBC's:

1. New Technical information relating to risks and safety procedures.
2. Serious accidents or releases involving recombinant DNA or RNA.
3. Serious illness of a laboratory worker which may be project related.
4. Other safety problems.

B. ANIMAL CARE

[ ] Project does not involve use of vertebrate animals.
[x] Project involves use of vertebrate animals. (Check the applicable statement).
  [ ] a) The project is in compliance with the Animal Welfare Act of 1966 and 9 CFR Subchapter A (Laboratory Animals), as amended.
  [x] b) This project is under review by the Institutional Care and Use Committee and a revised Form CSREES-662 will be submitted when the review is completed.
  [ ] c) This project has been approved by the Institutional Animal Care and Use Committee on ______________(Date).

C. PROTECTION OF HUMAN SUBJECTS

[x] Project does not involve use of human subjects.
[ ] Project involves use of human subjects. (Check the applicable statement).
  [ ] a) This project includes activities involving human subjects but can in no way be considered at risk. (If this statement is checked, the person signing this form must also initial in the space at right.)______________(Initial)
  [ ] b) This project is under review by an institutional committee as provided by our assurance and a revised Form CSREES-662 will be submitted when the review is completed.
  [ ] c) This project includes activities involving human subjects. Our institutional committee reviewed and approved it on ___________ in accordance with our assurance approved by S&E/and/or DHHS. The project will be subject to continuing review as provided for in that assurance.

SIGNATURE OF AUTHORIZED ORGANIZATIONAL REPRESENTATIVE	TITLE DR. JOHN J. O'NEIL ASSISTANT VICE CHANCELLOR RESEARCH DEVELOPMENT	DATE 5/31/2000

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB Approved 0524-003
Expires 5/0

National Environmental Policy Act Exclusions Form

Principal Investigator/Project Director Name: Robert A. Latour

Institution: Clemson University

Address: Office of Sponsored Programs, 300 Brackett Hall, Box 345702, Clemson University, Clemson, SC 29634-5702

Under 7 CFR Part 3407 (CSREES's implementing regulations of the National Environmental Policy Act of 1969 (NEPA) environmental data or documentation is required in order to assist CSREES in carrying out its responsibilities under NEPA which includes determining whether proposed research requires the preparation of an environmental assessment or a environmental impact statement, or whether such research can be excluded from this requirement on the basis of sever categories. Therefore, it is necessary for the applicant to advise CSREES whether the proposed research falls into one of th following Department of Agriculture or CSREES categorical exclusions, or whether the research does not fall into one o these exclusions (in which case the preparation of an environmental assessment or an environmental impact statement ma be required). Even though the applicant considers that a proposed project may or may not fall within a categorical exclusio CSREES may determine that an environmental assessment or an environmental impact statement is necessary for proposed project should substantial controversy on environmental grounds exist or if other extraordinary conditions o circumstances are present that may cause such activity to have a significant environmental effect.

Please Read All of the Following and Check All Which Apply

☐ **The proposed research falls under the categorical exclusion(s) indicated below:**

Department of Agriculture Categorical Exclusions
(found at 7 CFR 1b.3 and restated at 7CFR 3407.6 (a)(1)(i) through (vii))

☐ (i) Policy development, planning and implementation which are related to routine activities such as personnel, organizational changes, or similar administrative functions

☐ (ii) Activities that deal solely with the functions of programs, such as program budget proposals, disbursements, and transfer or reprogramming of funds

☐ (iii) Inventories, research activities, and studies such as resource inventories and routine data collection when such actions are clearly limited in context and intensity

☐ (iv) Educational and informational programs and activities

☐ (v) Civil and criminal law enforcement and investigative activities

☐ (vi) Activities that are advisory and consultative to other agencies and public and private entities, such as legal counseling and representation

☐ (vii) Activities related to trade representation and market development activities abroad

CSREES Categorical Exclusions
(found at 7 CFR 3407.6(a)(2)(i) through (ii))

The following categories of CSREES actions are exclude because they have been found to have limited scope an intensity and to have no significant individual or cumulativ impacts on the quality of the human environment:

(i) The following categories of research programs o projects of limited size and magnitude or with onl short-term effects on the environment:

☐ (A) Research conducted within any laborator greenhouse, or other contained facility wher research practices and safeguards preven environmental impacts

☐ (B) Surveys, inventories, and similar studies th have limited context and minimal intensity i terms of changes in the environment

☐ (C) Testing outside of the laboratory, such as i small isolated field plots, which involves th routine use of familiar chemicals or biologic materials

☐ (ii) Routine renovation, rehabilitation, or revitalizatio of physical facilities, including the acquisition an installation of equipment, where such activity i limited in scope and intensity

OR

☐ **Proposed research does *not* fall into one of the above categorical exclusions**
(**NOTE**: If checked, please attach an explanation of the potential environmental impacts of the proposed research. May require completion of an environmental assessment or an environmental impact statement.)

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

OMB Approved 0524-0033
Expires 5/00

National Environmental Policy Act Exclusions Form

Principal Investigator/Project Director Name Jesse L. Grimes	Institution North Carolina State University
Address Department of Poultry Science, Box 7608, Raleigh, NC 27695-7608	

Under 7 CFR Part 3407 (CSREES's implementing regulations of the National Environmental Policy Act of 1969 (NEPA)) environmental data or documentation is required in order to assist CSREES in carrying out its responsibilities under NEPA which includes determining whether proposed research requires the preparation of an environmental assessment or ar environmental impact statement, or whether such research can be excluded from this requirement on the basis of severa categories. Therefore, it is necessary for the applicant to advise CSREES whether the proposed research falls into one of the following Department of Agriculture or CSREES categorical exclusions, or whether the research does not fall into one of these exclusions (in which case the preparation of an environmental assessment or an environmental impact statement may be required). Even though the applicant considers that a proposed project may or may not fall within a categorical exclusion CSREES may determine that an environmental assessment or an environmental impact statement is necessary for a proposed project should substantial controversy on environmental grounds exist or if other extraordinary conditions o circumstances are present that may cause such activity to have a significant environmental effect.

Please Read All of the Following and Check All Which Apply

[x] The proposed research falls under the categorical exclusion(s) indicated below:

Department of Agriculture Categorical Exclusions
(found at 7 CFR 1b.3 and restated at 7CFR 3407.6 (a)(1)(i) through (vii))

[ ] (i) Policy development, planning and implementation which are related to routine activities such as personnel, organizational changes, or similar administrative functions
[ ] (ii) Activities that deal solely with the functions of programs, such as program budget proposals, disbursements, and transfer or reprogramming of funds
[ ] (iii) Inventories, research activities, and studies such as resource inventories and routine data collection when such actions are clearly limited in context and intensity
[ ] (iv) Educational and informational programs and activities
[ ] (v) Civil and criminal law enforcement and investigative activities
[ ] (vi) Activities that are advisory and consultative to other agencies and public and private entities, such as legal counseling and representation
[ ] (vii) Activities related to trade representation and market development activities abroad

CSREES Categorical Exclusions
(found at 7 CFR 3407.6(a)(2)(i) through (ii))

The following categories of CSREES actions are exclude because they have been found to have limited scope an intensity and to have no significant individual or cumulativ impacts on the quality of the human environment:

(i) The following categories of research programs c projects of limited size and magnitude or with onl short-term effects on the environment:
[x] (A) Research conducted within any laborator greenhouse, or other contained facility wher research practices and safeguards prever environmental impacts
[ ] (B) Surveys, inventories, and similar studies tha have limited context and minimal intensity i terms of changes in the environment
[ ] (C) Testing outside of the laboratory, such as i small isolated field plots, which involves th routine use of familiar chemicals or biologic materials
[ ] (ii) Routine renovation, rehabilitation, or revitalizati of physical facilities, including the acquisition an installation of equipment, where such activity limited in scope and intensity

OR

[ ] Proposed research does *not* fall into one of the above categorical exclusions
(**NOTE**: If checked, please attach an explanation of the potential environmental impacts of the proposed research. May require completion of an environmental assessment or an environmental impact statement.)

The Office for Sponsored Programs is committed to providing prompt proposal review. However, due to increased federal compliance requirements, we may be unable to process and approve your proposal on a RUSH basis if the issues listed below are not properly addressed prior to proposal submission. It is important that all supporting attachments, if requested, are included with the proposal submission.

Yes [✓ No] Does this project support K-12 teacher preparation/professional development/applied research?

Yes [✓ No] Did the Clemson University Development Office assist in this proposal?

Yes [✓ No] Does project require submission through CURF (Clemson University Research Foundation)? **If yes,** contact Office for Sponsored Pgms.

Yes [✓ No] Does sponsor's policy (federal or non-profit only) prohibit indirect costs? **If yes,** attach copy of policy.

Yes [✓ No] Does sponsor's policy require mandatory cost sharing or matching funds? **If yes,** attach copy of policy.

[✓ Yes] No Is proposal responding to a Request for Proposal (RFP) or program announcement? **If yes,** attach copy.

Yes [✓ No] Does project require foreign travel or employment? **If yes,** contact Human Resources (employment); Comptroller (travel)

Yes [✓ No] Does project involve permanent improvements, modifications or new facilities exceeding $100,000? **If yes,** contact FM&O.

Yes [✓ No] Does project involve federal classified, ITAR, or militarily-critical information?

Yes [✓ No] Is project likely to involve publication restrictions? **If yes,** circle: *BY SPONSOR* *BY CLEMSON*

Yes [✓ No] Does project involve confidential information or trade secrets? **If yes,** circle: *OF SPONSOR* *OF CLEMSON*

Yes [✓ No] Does project have intellectual property (patents) potential?

Yes [✓ No] Does project involve third party land or facility use? **If yes,** attach copy of supporting documentation.

Yes [✓ No] Does project include sub-contracted or consultant work? **If yes,** attach Statement of Work & Cost Estimate

Yes [✓ No] Does project involve radioisotopes or radiation-generating equipment? **If yes,** contact the Radiation Safety Officer.

Yes [✓ No] Does project involve environmental, health or safety issues? **If yes,** contact Environmental Health & Safety.

COMPLIANCE DATA

HUMAN SUBJECTS? (includes surveys and interviews)	**ANIMAL SUBJECTS?**	**BIOHAZARD/CHEMICAL?**	**RECOMBINANT DNA?**
Yes [✓ No]	[✓ Yes] No	Yes [✓ No]	Yes [✓ No]
Protocol Number	Protocol Number pending	Protocol Number	Protocol Number
Approval Date	Approval Date	Approval Date	Approval Date

CERTIFICATION AND APPROVAL SIGNATURES

Financial Disclosure: The undersigned certify that they have read, understand, and are bound by Clemson University's FINANCIAL DISCLOSURE policy, that they have made all financial disclosures required by it, if any, and will comply with any conditions or restrictions imposed by the institution to manage, reduce, or eliminate actual or potential **conflicts of interest**. Intellectual Property: The undersigned certify that they have read, understand, and are bound by Clemson University's patent policy and computer software copyright policy, agree to assign all rights, title, and interest in intellectual property under such policies to Clemson University, and to execute such further documents as needed to perfect the assignment of such rights. All investigators agree to disclose, and to cause other project personnel to disclose, all INTELLECTUAL PROPERTY to the University Intellectual Property Committee within 60 days of discovery or the time of confidential submission for publication of manuscripts disclosing the invention, whichever is earlier. Failure to make timely disclosure to the Committee may lead to the loss of patent rights to the federal government on federal government contracts or to other parties. Cost Accounting Standards: The principal investigator certifies that the proposed budget complies with Clemson University's federal cost accounting standards policy.

		Date
PRINCIPAL INVESTIGATORS		6/2/00 6/2/00
Dept Head/Director/Chair (as required by College)	6/2/00	6/2/00
Dean or School Official	School of Animal, Biomedical and Biological Sciences	6/5/00
CO-INVESTIGATORS	128 Long Hall Clemson University	
Dept Head/Director/Chair (as required by College)	Clemson, SC 29634-0304	6/2/00
Dean or School Official		

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE

APPLICATION FOR FUNDING

OMB Approved 0524-0022
Expires 7/31/01

FOR CSREES USE ONLY	
PROGRAM AREA CODE	PROPOSAL CODE

1. LEGAL NAME OF ORGANIZATION TO WHICH AWARD SHOULD BE MADE

Clemson University

3. NAME OF AUTHORIZED ORGANIZATIONAL REPRESENTATIVE

Y. T. Shah
Chief Research Officer and
Senior Vice Provost

4. a. PHONE NUMBER (Include Area Code) **864/656-2424**

b. FAX NUMBER **864/656-0881**

c. INTERNET ADDRESS **lrisour@clemson.edu**

2. ADDRESS (Give complete mailing address and Zip Code-including County)

Sponsored Programs
300 Brackett Hall, Box 345702
Clemson University
Clemson, SC 29634-5702 **Pickens County**

5. ADDRESS OF AUTHORIZED ORGANIZATIONAL REPRESENTATIVE (If different from Item 2.)

6. TITLE OF PROPOSED PROJECT (80-character Maximum, including spaces)

Adhesin-Specific Nanoparticles for Removal of *Campylobacter jenjuni* from Poultry

7. PROGRAM TO WHICH YOU ARE APPLYING (Refer to Federal Register Announcement where applicable)

Integrated Research, Education, Extension Competitive Grants rogram—National Food Safety Initiative

8. PROGRAM AREA AND NUMBER (Refer to Federal Register Announcement where applicable)

111.2

9. IRS NO.	10. CONGRESSIONAL DISTRICT NO.	11. PERIOD OF PROPOSED PROJECT DATES	12. DURATION REQUESTED
57-6000254	**SC 3rd**	From: **01/01/01** Through: **12/31/03**	

13. TYPE OF REQUEST (Check only one)

☒ New ☐ Renewal ☐ Supplement ☐ Resubmission
☐ Continuing Increment ☐ PI Transfer [PRIOR USDA Award No. ____________]

14. FUNDS REQUESTED (From Form CSREES-55)

$599,715

15. PRINCIPAL INVESTIGATOR(S)/PROJECT DIRECTOR(S)

a. PI/PD #1 Name (First,Middle,Last) SS #* (Correspondent PI)

Robert A. Latour 160-52-0700

b. PI/PD #2 Name (First,Middle,Last) SS #*

Fred J. Stutzenberger 407-60-3565

c. PI/PD #3 Name (First,Middle,Last) SS #*

16. a. PI/PD #1 PHONE NUMBER (Include Area Code):

b. FAX NUMBER: **864/656-5552**

c. INTERNET ADDRESS: **864/656-4466**

17. PI/PD #1 BUSINESS ADDRESS (Include Department/Zip Code)

Department of Bioengineering
501 Rhodes, Boc 340905
Clemson University
Clemson, SC 29634-0905

***Submission of the Social Security Number is voluntary and will not affect the organization's eligibility for an award. However, it is an integral part of the CSREES information system and will assist in the processing of the proposal.**

18. TYPE OF PERFORMING ORGANIZATION (Check one only)

01 ☐ USDA/S&E Laboratory
02 ☐ Other Federal Research Laboratory
03 ☐ State Agricultural Experiment Station (SAES)
04 ☒ Land-Grant University 1862
05 ☐ Land-Grant University 1890 or Tuskegee University
06 ☐ Private University or College
07 ☐ Public University or College (Non Land-Grant)
08 ☐ Private Profit-making
09 ☐ Private Non-profit
10 ☐ State or Local Government
11 ☐ Veterinary School or College
12 ☐ 1994 Institution
13 ☐ Individual
14 ☐ Other (Specify)
15 ☐ Hispanic-serving Institution

19. WILL THE WORK IN THIS PROJECT INVOLVE RECOMBINANT DNA?

☒ No ☐ Yes (If yes, complete Form CSREES-662)

20. WILL THE WORK IN THIS PROJECT INVOLVE LIVING VERTEBRATE ANIMALS?

☐ No ☒ Yes (If yes, complete Form CSREES-662)

21. WILL THE WORK IN THIS PROJECT INVOLVE HUMAN SUBJECTS?

[X] No [ ] Yes (If yes, complete Form CSREES-662)

22. WILL THIS PROJECT BE SENT OR HAS IT BEEN SENT TO OTHER FUNDING AGENCIES, INCLUDING OTHER USDA AGENCIES?

[X] No [ ] Yes (If yes, list Agency acronym(s) & program(s))

By signing and submitting this proposal, the applicant is providing the required certifications set forth in 7 CFR Part 3017, as amended, regarding Debarment and Suspension and Drug-Free Workplace; and 7 CFR Part 3018 regarding Lobbying. Submission of the individual forms is not required. (Please read the Certifications and Instructions included in this kit before signing this form.)

In addition, the applicant certifies that the information contained herein is true and complete to the best of its knowledge and accepts as to any award, the obligation to comply with the terms and conditions of Cooperative State Research, Education, and Extension Service in effect at the time of the award.

SIGNATURE OF PRINCIPAL INVESTIGATOR(S)/PROJECT DIRECTOR(S) (All PI(s)/PD's listed in block 15 must sign if they are to be included in award document.)

DATE 6/2/00 6/2/00

SIGNATURE OF AUTHORIZED ORGANIZATIONAL REPRESENTATIVE (Same as Item 3)

TITLE Chief Research Officer and Sr. Vice Provost

DATE 6/6/00

APPENDIX E

CLEMSON
UNIVERSITY

MEMORANDUM

Date: November 20, 2000

To: Fred Stutzenberger, Robert A. Latour, Jr.,
Ya-Ping Sun, Tzuen-Rong J. Tzeng

From: Stephen R. Chapman

RE: **Patent Disclosure Related to Nanoparticles**

Thank you for your patience in explaining the technology of your invention. As we discussed and agreed, the concept has apparently enormous potential. At our last meeting, we agreed that prior to formal submission for consideration by the patent committee more specific findings are required on which to base an application and justify claims.

Your invention is, in my opinion, patentable, to important to merely put on hold. I recommend that a presentation be made to a subcommittee, the usual starting point, to affirm an institutional commitment to your work and re-examine what work might be needed.

By copy of this memo, I am asking Janet Dillon to contact Fred and discuss appropriate meeting times and the best committee.

I look forward to seeing your work mature into a scientifically and economically viable effort. As we discussed, I will work with you to move it forward as rapidly as possible. I "leave the scene" when my efforts become a delay!

1889

SENIOR CONTRACT ADVISOR
300 Brackett Hall Box 345701 Clemson, SC 29634-5701
864.656.1296 FAX 864.656.1498

APPENDIX F

864-656-5026 YA PING

(PROTOCOL.Janna1.doc)

IACUC # 00-050-A
Date Approved Dec 7, 2000
Date Expires Dec 6, 2003

DEPARTMENT OF POULTRY SCIENCE
NORTH CAROLINA STATE UNIVERSITY
RESEARCH PROTOCOL

1. **TITLE:** Effect of Polystyrene Cores on Poult Performance

2. **SPONSOR:** NC06390

3. **SHORT EXPERIMENT CODE NAME:** (NC06390)

4. **CHARGE COSTS TO:** Jesse Grimes (NC06390)

5. **LOCATION:** Lake Wheeler Field Laboratory
Turkey Educational Unit - House 287, pens 61-68

6. **PLACEMENT DATES:** September 5, 2001

7. **TERMINATION DATES:** December 14, 2001

8. **PROJECT LEADERS:** J.L. Franklin, J.L. Grimes, B.W. Sheldon, and M.J. Wineland

9. **NUMBERS OF BIRDS ASSIGNED BY HOUSE AND ROOM:**
240 poults, 8 pens of birds

10. **LIST OF TOXIC SUBSTANCES INVOLVED:** NA

11. **SAFETY PRECAUTIONS REQUIRED:**
proper safety procedures for euthanasia of cull and sick birds

12. **PROJECT INITIATION RESPONSIBILITIES:**

	RESPONSIBLE (x)		
	PI	LAT/FL	N/A
Order ingredients		x	
Order feed		x	
Deliver feed or ingredients		x	
Order chicks or poults	x		
Deliver chicks or poults		x	
Arrange for sexing			x
Placement and banding	x	x	
Arrange for vaccine(s)			x
Standard facility prep		x	

DEPARTMENT OF POULTRY SCIENCE, RESEARCH PROTOCOL

18. BASIC EXPERIMENTAL DESIGN:
Birds will be gavaged at one week of age. Doses of 0.1, 0.5, and 1.0 ml per bird of the 10 polystyrene core samples will be administered to three birds per pen. There will be six to eight pens of birds. Each pen will contain 33 birds (10 treatments + control x 3).

19. UNUSUAL CRITICAL PROCEDURAL STEPS REQUIRING SPECIAL ATTENTION:
provide proper identification of pens

20. FEED MIXING DATES: as needed

21. FEED CHANGING AND FEED WEIGHBACK DATES:

Starter 1 to 3 weeks
Starter 2 to 6 weeks
Grower 1 to 8 weeks
Grower 2 to 10 weeks
Finisher 1 to 12 weeks
Finisher 2 to 14 weeks

22. SCHEDULE OF BIRD WEIGHING DATES:
September 12, 26, and October 17

23. LIGHTING PROGRAM SCHEDULE:

Calendar Wk.	Wk. of Age	Hours & Minutes of Light	Intensity Light Type
September 5	Week 1	24 hours	
	Weeks 2-14	Natural light	

24. HOUSE OR ROOM TEMPERATURE:
Background temperature: 75-80 F. A heat lamp will be utilized for chicks.

25. VACCINE TYPES AND VACCINATION DATES:
None

26. WINGBAND, LEGBAND OR OTHER BIRD IDENTIFICATION PROCEDURES:
birds will be identified by wing bands

27. HANDLING OF BIRDS FOUND OUT OF PEN OR CAGE:
replace by wing band number to correct pen

28. LITTER REQUIREMENTS: none

29. EGG COLLECTION PROCEDURES:

30. SPECIAL EGG SAMPLING DATES:

31. HATCHING EGGS:

32. BIRD MOVEMENT DATES:

DEPARTMENT OF POULTRY SCIENCE, RESEARCH PROTOCOL

33. FEEDING PROGRAM: Feed is to be provided *ad libitum.*

Starter 1 to 3 weeks
Starter 2 to 6 weeks
Grower 1 to 8 weeks
Grower 2 to 10 weeks
Finisher 1 to 12 weeks
Finisher 2 to 14 weeks

34. WATER RESTRICTION PROGRAM:
water is to be provided ad libitum

35. DIETS: (ATTACHED SIGNED MIX SHEETS)
see attached feed sheets

36. PROCEDURE FOR REMOVING INJURED OR CRIPPLED BIRDS FROM THE EXPERIMENT:
Remove and weigh bird, euthanize birds, record on data sheet

37. PROCEDURE FOR THE TERMINATION OF THE PROJECT AND DISPOSAL OF THE RESEARCH ANIMALS:
Birds will reared to 14 weeks of age and then be either sold to a processor, euthanized, or transferred to another faculty for their use.

38. BLOOD SAMPLE SCHEDULE: N/A

39. NECROPSY SCHEDULE AND PROCEDURES:
If necessary, sick birds will be sent to Rollins lab for necropsy.

40. SPECIAL REQUIREMENTS AND PROCEDURES: NA

41. TISSUE SAMPLING PROCEDURES & LABORATORY ANALYSES: NA

42. UNIVERSITY REQUIREMENTS:
The principal investigator verifies all relevant University Guidelines are being met and project protocols were approved by the relevant committees.

Radiological Safety ______________________ Project # __________
Date approved

Biological Hazards ______________________ Project # __________
Date approved

Principal Investigator ____________________ Date ______________
____

Approved: ______________________________ Date ______________
____

APPENDIX G

1/3/2001

To: Dr. Fred Stutzenberger
Dept. of Microbiology

From: Diann Tinsley
ABBS Business Center

Please check the following budget on your award from USDA. If there are questions or if you see discrepancies, please contact me.

PI: FJS

Title: ADHESION-SPECIFIC NANOPARTICLES FRO REMOVAL OF CAMPYLOBACTER JEJUNI FROM POULTRY

Sponsor: USDA

Beg Date: 9/14/2000

End Date: 9/15/2004

Funds:

Uclass Sal	[redacted]
Grad Stud	[redacted]
Fringe	[redacted]
Travel	[redacted]
Other (supplies)	[redacted]
Total	[redacted]

Budget: Submitted vs. Revised	Year 1 Proposed	Yr. 1 Revised	Year 2 Proposed	Yr. 2 Revised	Year 3 Proposed	Yr. 3 Revised	Total Proposed	Total Revised
Personnel								
Bob Latour, Co-PI (1 mo.)	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Fred Stutzenberger Co-P.I. (1 mo.)	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Ya-Ping Sun, Co-Inv. (0 mo.)	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
John Rodgers, Co-Inv. (1 mo.)	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Fringe (18%)	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
T.-R. Tzeng, Co.Inv. (1,2,2 mo.)	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Fringe (26%)	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Grad Student (3,4,4@16k)	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Fringe (0.8%)	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Total Personnel	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Equipment	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Supplies								
Nanoparticle synthesis	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
In vitro bioactivity testing	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Toxicity testing	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Histopathology	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Scanning electron microscopy	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Misc. lab supplies (1000/student)	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Communication (mail,fax,phone)	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Reference materials	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Total Supplies	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Travel	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Publication	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Subcontract (NC State)	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Direct Cost	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
Indirect Cost (19% of total)	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
TOTAL	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
TARGET = Original Budget - 10%:		[redacted]		[redacted]		[redacted]		[redacted]

APPENDIX H

Annul Report:

Introduction:

The work during the whole year (From the middle October 2000 to the middle October 2001) will be divided into 6 periods.

1. Period 1: From the very beginning to the end of the fall 2000, this period was mainly used in book, paper searching and reading, and accumulated information needed for culture and experimental handle the bacteria of *C. jejuni*. Gave the presentation about the *C. jejuni* at the end of fall 2000. (Please refer to the **Appendix I).**

2. Period 2: (before 07/03) During this period of time, got the bacteria strain of CJ 23, CJ46, CJ49, CJ71 and CJ72. Tried to culture and verify that there were *C.jejuni*. But at last, sent email to ask for information on Jan. 25 (please refer to the **appendix II**), there were not *C. jejuni.* In February, Dr. Tzeng decided to order the ATCC strain of *C.jejuni.* Got the ATCC bacteria strain. Did TEM (**Appendix III**) to verify. This period was used mainly to solve the "Reducing sugar determination by the Bernfeld method" and the "Uptaking *versus* Adhesion" of the sugar.

3. Period 3: The Chemistry Department sent some "chocolate milk" (fluorescent-PEG-nanoparticle, 250nm, 900nm) after the first monthly Three-group-meeting on Feb. 13, 2001. Did some experiments in this batch of nanoparticles.

4. Period 4: (07/03—8/18) On July 3rd, 2001, the first batch of PEG-core-nanoparticles and mannose functionalized (Cat #, Shelby 6-20-02) were delivered by the Chemistry. Tried to do some experiments. In this period of time, got the poultry strains of B_5CD_6, B_5CD_{30}, Ce0-3, and Ce1-9. Cultured and did SEM (**Appendix IV**) to verify them. Also, in this period, tried to get bacteria and nanoparticle binding SEM pictures (**Appendix V**).

5. Period 5: (08/20—08/29) On Aug. 18th, 2001, got the batch of W.J. P109 (7-17-01) nanoparticles. Slide Agglutination test and Viable Counting Experiment.

6. Period 6: (08/29---Today) On Aug. 29th, 2001, got two batches of nanoparticles of Q-26 Pst-mannose and Weijie 8-24-P141 PS-mannose. Did some experiments of Slide agglutination and Viable counting test. Also finished the Growth Curve of Poultry B_5CD_{30}.

Period 1:

Paper searching and reading, gave presentation about the literature review. Please refer to the appendix I to read the presentation paper.

Period 2:

Mainly solve the problem of Mannose binding or Uptaking problem.

(June1) Reducing sugar determination by the Bernfeld Method

Material needed:

1. Reagents: 1mg/ml mannose, 1 M phosphate buffer, Bernfeld reagent.
2. Bacteria: Phosphate buffered *C. jejuni* of CJ 49.
3. Instruments: Ice bath, Water bath, Spectronical 20, Syringe, Whatman 0.45μm pore size Acrodisc.

Prepare the bacteria:

1. Centrifuge the liquid bacteria, suspend by using 1 M phosphate buffer. Centrifuge again and re-suspend.
2. Measure the Optical Density (Spectronical 20) of the re-suspended bacteria. OD_{600}nm=0.8

The experiment:

1. 1ml of the bernfeld reagent to a series of test tubes add 0.5 ml distilled water to each tube.
2. Put the test tubes on the ice bath.
3. Serially add 25,50,75,100,150,200,250ul of 1mg/ml mannose to the test tubes.
4. Serially add 475, 450,425,400,350,300, 250ul of prepared of the *C.jejuni* to the tubes.
5. Filter the samples obtained after the bacteria and mannose mixed.
6. Blank: add 0.5 ml of water to one tube to sever as blank.
7. Boil all the tubes for 10 min. Cool and add 1 ml of distilled water.
8. Read all tubes against the blank (step 3) at 540nm.

9. Calculate mannose equivalents in each sample versus the mannose standard curve.

The results:

.712 = 500μg

Mannose (1mg/ml)	μl dH2O	[Conc.] μg / ml	OD_{540nm} under ice bath	OD_{540nm} w/o ice bath
0	500	0	0	0
25	475	50	0.024	0.015
50	450	100	0.079	0.063
75	425	150	0.147	0.108
100	400	200	0.237	0.154
150	350	300	0.381	0.219
200	300	400	0.452	0.291
250	**250**	500	0.682	0.431

(June/03) Uptaking *versus* adhesion

Purpose:

In order to determine whether the mannose is uptaken by the bacteria or is bound to the bacteria. Set up this experiment.

Material needed:

1. Reagents: 1mg/ml mannose, 1 M phosphate buffer, Bernfeld reagent.
2. Bacteria: Phosphate buffered *C. jejuni*
3. Instruments: Ice bath, Water bath, Spectronical 20, Syringe, Whatman 0.45μm pore size Acrodisc.

Prepare the bacteria:

1. Centrifuge the liquid bacteria, suspend by using 1 M phosphate buffer. Centrifuge again and re-suspend.
2. Measure the Optical Density (Spectronical 20) of the re-suspended bacteria.

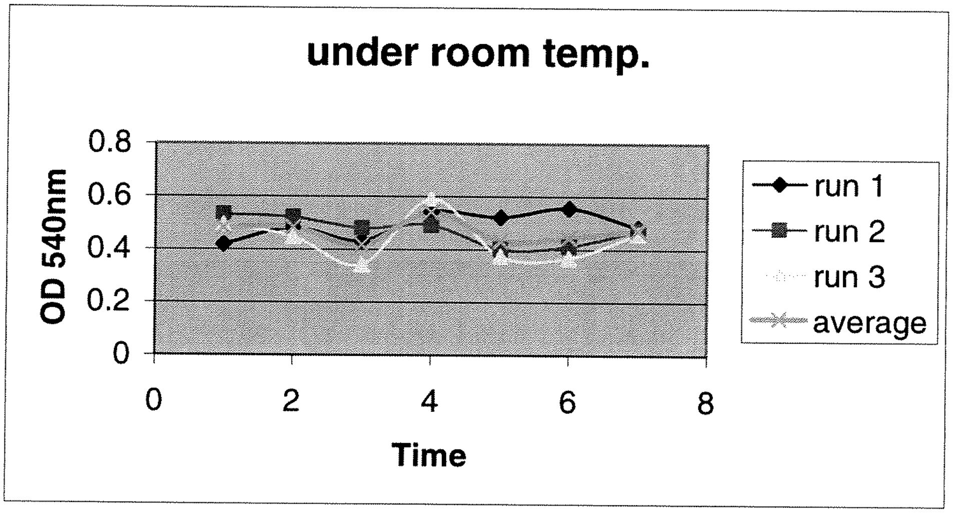
Procedures:

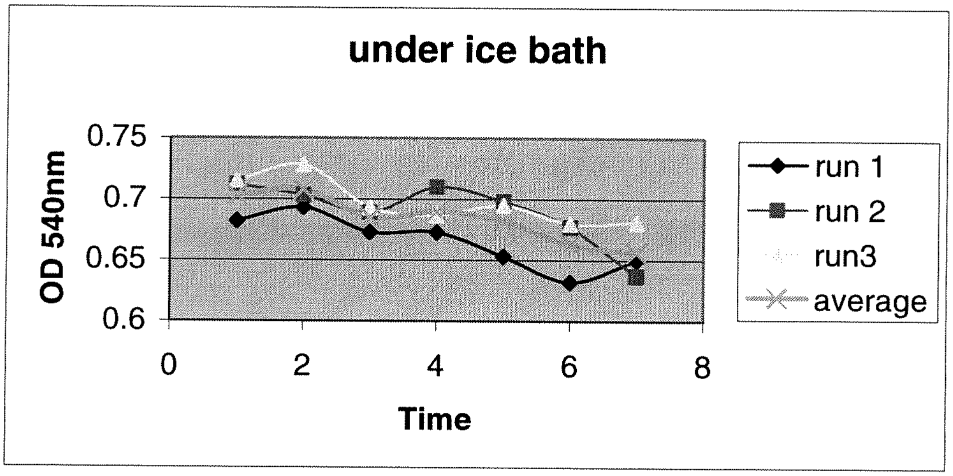
1. Add 1 ml of Bernfeld reagent to a series of test tubes (15 X 150mm). Add 0.5 ml distilled water to each tube.
2. Using the ice bath prepared, add 250ul of mannose (1mg/ml) and 250ul of prepared bacteria to the series of test tubes.

3. Blank: add 0.5 ml of water to one tube to sever as blank.
4. Filter the samples (obtained from step 2) after the bacteria and mannose mix together for 15", 30", 45", 60" …
5. Boil all the tubes for 10 min.
6. Boil all the tubes for 10 min. Cool down and add 1 ml of distilled water.
7. Read all tubes against the blank (step 3) at 540 nm
8. Calculate mannose equivalents in each sample versus the mannose standard curve.

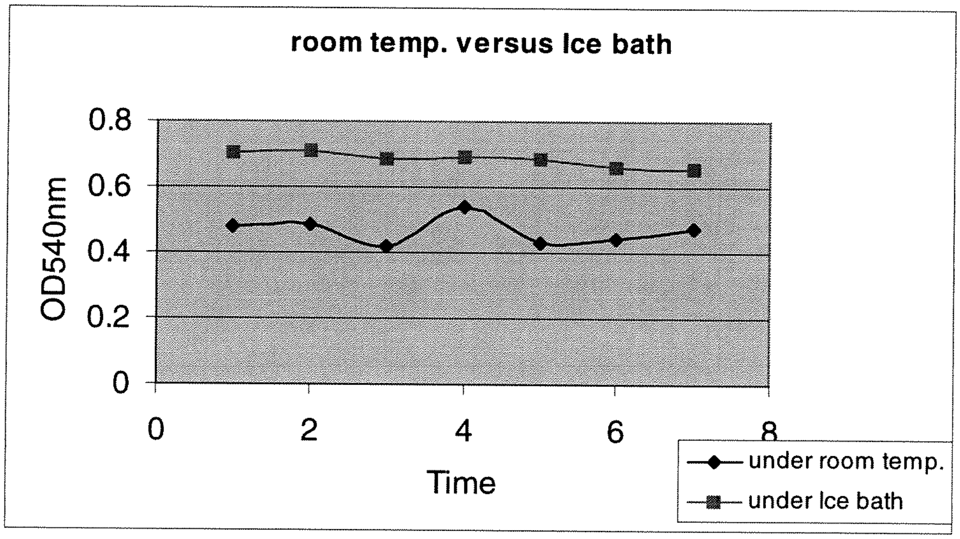
Results:

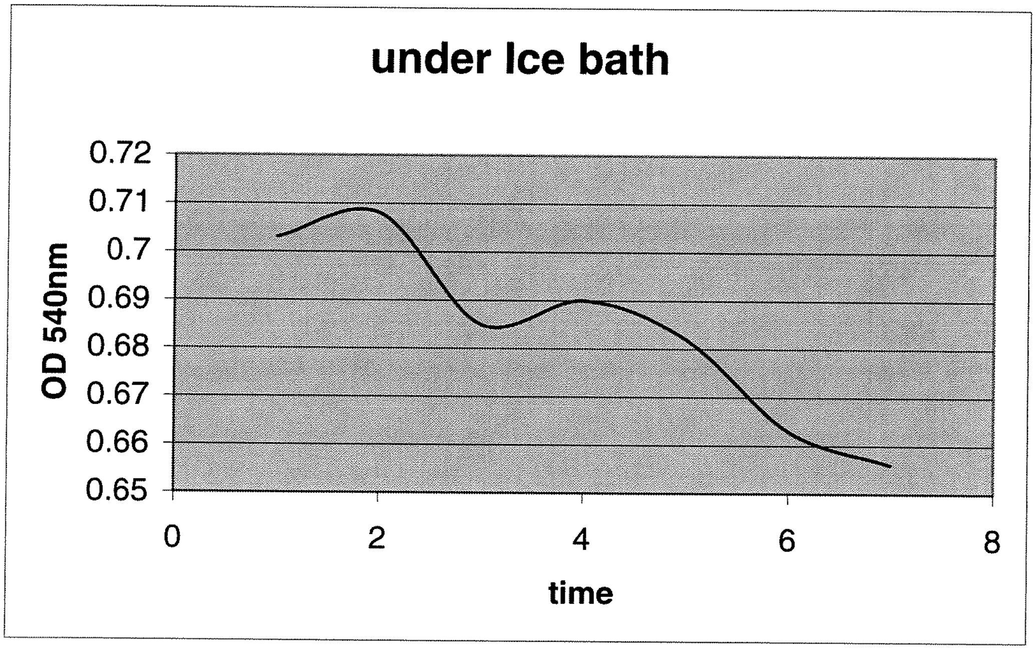
Time (second)	Under room temp.			Under ice bath				
	Run 1	Run2	Run3	Run 1	Run 2	Run 3	0.712	500
15"	0.418	0.532	0.493	0.682	0.712	0.715	703	494
30"	0.481	0.521	0.447	0.693	0.703	0.728	.708	497
45"	0.432	0.48	0.342	0.673	0.689	0.693	.685	481
60"	0.541	0.491	0.591	0.673	0.71	0.687	.690	484
75"	0.521	0.401	0.374	0.653	0.698	0.695	.682	478
90"	0.556	0.412	0.367	0.632	0.677	0.68	.663	465
105"	0.483	0.472	0.458	0.65	0.637	0.681	.655	459

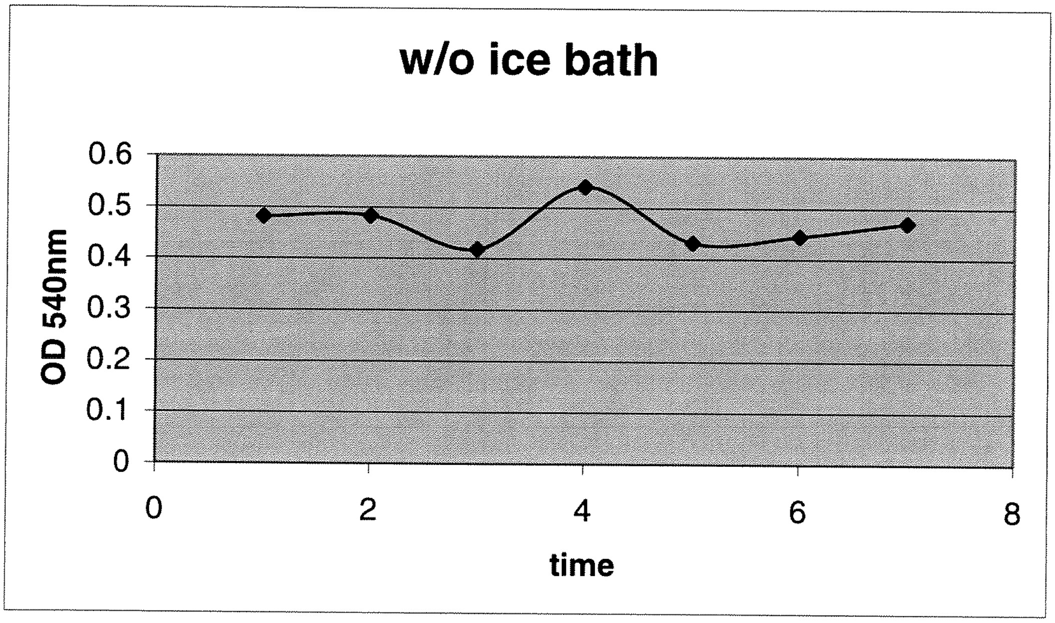




Time (seconds)	15"	30"	45"	60"	75"	90"	105"
Room temperature	0.481	0.483	0.418	0.541	0.432	0.445	0.471
Under ice bath	0.703	0.708	0.685	0.69	0.682	0.663	0.656




under Ice bath
0.72
0.71
0.7
0.69
0.68
0.67
0.66
0.65
OD 540nm
0
2
4
6
8
time


w/o ice bath
0.6
0.5
0.4
0.3
0.2
0.1
0
OD 540nm
0
2
4
6
8
time

Transmission Electron Microscopy

Material and methodology:

A coated grid was floated on a droplet of ATCC stain of *C. jejuni* on parafilm for 1 minute to permit adsorption of the specimen. The grid was then transferred onto a nearby drop of phosphotungistic acid (PTA) for stain for 30 seconds. Any excess liquid was blotted using a piece of filter paper, and then air-dried for 30 minutes. It was then examined under the Transmission Electron Microscopy. Pictures were taken for future analysis.

Results:

Please refer to the appendix III.

Period 3:

Got the first batch of the PEG-Core Fluorescent nanoparticle (Chocolate milk). Tried to do some experiment to see if there is any bind between the nanoparticle and the ATCC strain of *C. jejuni.*

Nanoparticle slide agglutination

Material needed:

1. Microorganism: ATCC strain of *C. jejuni* (measure $OD_{600nm} = 0.7$. serially dilute 1:1, 1:2, 1:4, 1:8, 1:16…)
2. Reagents: Non-functionalized nanoparticles with PEG chain (250nm), non-functionalized nanoparticles with PEG chains (900nm). ("chocolate milk" (fluorescent-PEG-nanoparticle)
3. Instruments: Microscopy slides, sterilized toothpick, light microscopy.

Material preparation:

1. Microorganism: ATCC strain *C.jejuni* from liquid culture, centrifuge, PBS buffer suspended, measure $OD_{600nm} = 0.5$, serially dilute the *C.jejuni* (1:2, 1:4, 1:8, 1:16, 1:32…).
2. Nanoparticles: serially dilute the 250nm PEG-Core nanoparticle solution to 10^{-1}, 10^{-2}, 10^{-3},10^{-4}
3. Serially dilute the 900nm PEG-Core nanoparticle solution to 10^{-1}, 10^{-2}, 10^{-3},10^{-4}

Experiment:

1. Take one drop of the original concentration of the bacteria on slide.
2. Take one drop of 250nm non-functionalized nanoparticles on one of the slides.
3. Use a toothpick to mix the drops.
4. Exam visually unaided for agglutination.
5. Exam under microscopy for agglutination.
6. Repeat the experiment by using different conc. of bacteria (obtained from material preparation) and different proportional nanoparticles (obtained from material preparation).
7. Repeat the experiment by using 900nm PEG-Core nanoparticles.

Result: (without functionalized nanoparticles).

Can't see any agglutination by naked eyes and under light microscopy at various concentrations of the cells and 250nm PEG-core nanoparticles.

	1:2	1:4	1:8	1:16	1:32
10^{-1}	No	No	No	No	No
10^{-2}	No	No	No	No	No
10^{-3}	No	No	No	No	No
10^{-4}	No	No	No	No	No

Can't see any agglutination by naked eyes and under light microscopy at various concentrations of the cells and 900nm PEG-core nanoparticles.

	1:2	1:4	1:8	1:16	1:32
10^{-1}	No	No	No	No	No
10^{-2}	No	No	No	No	No
10^{-3}	No	No	No	No	No
10^{-4}	No	No	No	No	No

Viable counting

Purpose:

To determine if the PEG-Core-nanoparticles ("chocolate milk" fluorescent-PEG-nanoparticle) have the ability to bind the bacterial cells, use viable counting experiment.

Methods:

1. Take cultured 2-day bacteria, centrifuge; suspended by PBS buffer; re-centrifuged, re-suspended. Standardize OD to OD_{600}nm=0.985 by Spectronic 20.
2. Using aseptic techniques, make 10^{-2}, 10^{-4}, 10^{-6}, 10^{-7} dilution. Make 10^{-2}, 10^{-4}, 10^{-6}, dilution by serially diluting 1ml of culture with 99 ml buffer. 10^{-7} by using 1ml 10^{-6} dilution and 9ml buffer.
3. Using spread plate techniques, for each concentration of bacteria, plate out, in duplicate, by spreading 0.1ml of each dilution on plate.
4. Take 0.1ml of the 10^{-2} conc. Of the bacteria, mix with 0.1ml of the 900 nm PEG-core-nanoparticles. After about 30 seconds, plate out, in duplicate, by spread plate technique.
5. Repeat step 4 by using 10^{-4}, 10^{-6}, 10^{-7} conc. of the bacteria.
6. Seal in to BBL Campy Pouch Microaerophilic System, put into incubator.
7. Exam after 24 and 48 hours.

Results:

Exam the colonies after 24 hours								
	10^{-2}		10^{-4}		10^{-6}		10^{-7}	
Bact only	TMTC	TMTC	139	166	7	3	0	0
nano+bact	TMTC	TMTC	152	167	3	2	0	0

Exam the colonies after 48 hours								
	10^{-2}		10^{-4}		10^{-6}		10^{-7}	
Bact only	TMTC	TMTC	148	176	8	3	0	0
nano+bact	TMTC	TMTC	172	185	4	2	0	0

Repeat the June-14 experiment under the same experiment conditions on June 19, 2001.

Exam the colonies after 24 hours						
	-2		-4		-6	
Bact only	TMTC	TMTC	91	104	1	0
nano+bact	TMTC	TMTC	124	54	1	0

Exam the colonies after 48 hours						
	-2		-4		-6	
Bact only	TMTC	TMTC	98	104	2	0
nano+bact	TMTC	TMTC	128	58	1	0

Since the results of June-14 and June-19 are different. Used the same bacteria and the same experiment conditions. Repeat the experiment on **June-22-2001.**

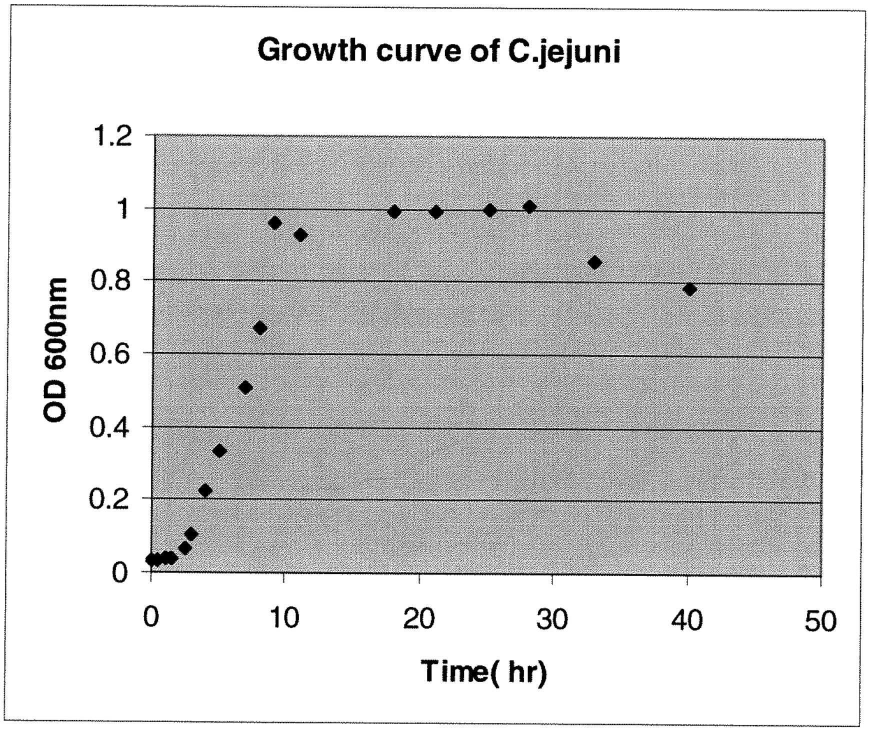
Exam the colonies after 24 hours						
	-2		-4		-6	
Bact only	118	232	38	86	1	1
nano+bact	123	152	56	32	0	2

Exam the colonies after 48 hours						
	-2		-4		-6	
Bact only	120	231	38	86	1	1
nano+bact	128	154	56	33	0	2

Growth Curve of ATCC strain of *C.jejuni* *(may be contaminated)*

Bacterial ATCC *C.jejuni* grew in Brucella Broth under 43℃ for 18 hours, then transfer 1.5ml to about 120ml Brucella broth liquid media, divided the 120ml media to 40 test tubes, each on contained 3ml. Put into Campy Pouch. Incubated under 42-43 ℃. Every a few hours, took a sample and measure the OD.

The Growth Curve



Period 4:

In this period of time, the first batch of PEG-core-nanoparticles and mannose functionalized (Cat #, Shelby 6-20-02) were delivered by the Chemistry on July-3-2001.

(done) Slide agglutination test:(07/03)

Materials:

1. After cultured for 2 days, ATCC strain of *C. jejuni* was centrifuged and resuspended to OD $_{600}$nm=0.6
2. 250nm non-functionalized nanoparticles of batch Shelby 6-20-02.
3. 250nm functionalized nanoparticles (Cat #, Shelby 6-20-02)

Methods: (refer to Page 7)

1. Serially dilute *C.jejuni* to 10^{-5} 10^{-6},
2. Serially dilute non-functionalized nanoparticles and functionalized nanoparticles to 1/2, 1/4, 1/8. 1/16, 1/32
3. Mix various conc. of nanoparticles with various conc. of bacteria on the slides.
4. Exam under unaided eyes.
5. Exam under light microscopy 100X, 400X,

Results:

No apparent Agglutination in whatever mixture no matter under unaided eyes or under light microscopy for non-functionalized nanoparticles.

	1:2	1:4	1:8	1:16	1:32
10^{-1}	No	No	No	No	No
10^{-2}	No	No	No	No	No
10^{-3}	No	No	No	No	No
10^{-4}	No	No	No	No	No
10^{-5}	No	No	No	No	No
10^{-6}	No	No	No	No	No

No apparent Agglutination in whatever mixture no matter under unaided eyes or under light microscopy for mannose-chain nanoparticles.

	1:2	1:4	1:8	1:16	1:32
10^{-1}	No	No	No	No	No
10^{-2}	No	No	No	No	No
10^{-3}	No	No	No	No	No
10^{-4}	No	No	No	No	No
10^{-5}	No	No	No	No	No
10^{-6}	No	No	No	No	No

Viable counting

Material:

1. ATCC strain of *C. jejuni.*
2. Functionalized Nanoparticles (batch of Shelby 6-20-02).
3. Non-functionalized nanoparticles (batch of Shelby 6-20-02, this one is got together with_Shelby 6-20-02).
4. Brucella broth agar plates,

Methods: (refer to page 8)

1. Prepare *C. jejuni*; centrifuge, resuspended by PBS buffer. Measure OD_{600}nm= 0.8
2. Serially dilute to10^{-3} 10^{-4} 10^{-5} 10^{-6}.
3. Take 0.1ml of the above cell suspension, in duplicated, plate them out by spread plate techniques.
4. Take 0.1ml of functionalized nanoparticles, mix with 0.1ml of 10^{-3} bacterial dilution for 30 seconds. Then plate them out.
5. Repeat step 4 by using 10^{-4}, 10^{-5} 10^{-6} conc. of the bacteria.
6. Use Non-functionalized nanoparticles as control.
7. Seal in to BBL Campy Pouch Microaerophilic System, put into incubator.
8. Culture 24 and 48 hours then count the colonies.

Results:

7/4/2001, Viable counting for (Cat.# Shelby 6-20-02) after 48 hours

	10^{-3}		10^{-4}		10^{-5}		10^{-6}	
Bact. only	N/A	N/A	36	9	3	1	1	0
Fun. Nano	N/A	N/A	33	36	11	5	0	3
Non-fun-nan0	N/A	N/A	9	2	0	3	0	0

Page 24, 07-06-2001

Viable counting:

Repeat the viable counting experiment (07/04) of nanoparticles of (**Cat. # Shelby 6-20-02**).

Methods: (refer to page 8)

1. Prepare *C. jejuni*; centrifuge, resuspended by PBS buffer. Measure OD_{600nm}= 0.8
2. Serially dilute to10^{-3} 10^{-4} 10^{-5} 10^{-6}.
3. Take 0.1ml of the above cell suspension, in duplicated, plate them out by spread plate techniques.
4. Take 0.1ml of functionalized nanoparticles, mix with 0.1ml of 10^{-3} bacterial dilution for 30 seconds. Then plate them out.
5. Repeat step 4 by using 10^{-4}, 10^{-5} 10^{-6} conc. of the bacteria.
6. Use Non-functionalized nanoparticles as control.
7. Seal in to BBL Campy Pouch Microaerophilic System, put into incubator.
8. Culture 24 and 48 hours then count the colonies.

Results:

7/6/2001, Viable counting for (Cat.# Shelby 6-20-02) after 24 hours

	10^{-3}		10^{-4}		10^{-5}		10^{-6}	
Bact.only	TMTC	TMTC	TMTC	TMTC	185	212	19	20
fun. Nano	TMTC	TMTC	TMTC	TMTC	172	200	13	26
non-fun-nan0	TMTC	TMTC	TMTC	TMTC	170	186	13	14

July-10.

Purpose:

To determine if there is any bacteria in the nanoparticles which can grow under the Microaerophilic conditions. (if there will be any contamination to the bacteria during the viable or other experiment.)

Methods:

1. Take 0.1ml functionalized nanoparticles, spread out, in duplicate, on the BB agar plates, culture under microaerophilic condition.
2. Take 0.1ml non-functionalized nanoparticles, spread out, in duplicate, on the BB agar plates, culture under microaerophilic condition.
3. Seal in the pouch, and incubate for 24 and 48 hours.

Results:

For each plate, there is no colony growth.

Titration experiment:

Methods:

ATCC strain of bacteria *C. jejuni*, Centrifuge, suspend and standardized to $OD_{600nm}=0.8$. Divided into several test tubes. In the first test tube, add $100\mu l$ of 10^{-2} functionalized nanoparticles, shake the test tube to see if there is any agglutination. If can't see any agglutination, add one more 100ul of the nanoparticles to increase the concentration of the nanoparticle…then…gradually, increase the concentration of the nanoparticles.

Results:

Couldn't see any agglutination in this experiment.

July 10 to July 13.

Scanning Electron Microscopy

Prepare specimen, including bacteria only, functionalized nanoparticle only, Non-functionalized nanoparticle only, Mixture of Bacteria and Nanoparticles,

Procedures:

1. Fixation: Use 3.5% of the cacodylate buffered glutaraldehyde at PH 7.2 for 24 hours at 4 °C
2. Dehydration: after removal from the fixative, samples will be dehydrate in ethyl alcohol with increasing concentration for certain period of time.

   50% ETOH------------ 15 min
   70% ETOH ------------- 15min
   85% ETOH ------------- 15min
   95% ETOH -------------15 min
   100% ETOH --------------30min
   100% ETOH--------------- 30min

3. Critical point dry, after dehydration, a pointer dryer will be used for critical point drying of the samples.
4. Mount: samples will be mounted on Aluminum stubs
5. Coat: A Hummer X sputter coater was used to coat mounted the stubs with gold to an approximate thickness of 5nm.
6. Exam under the SEM. Samples were examined in a Scanning Electron Microscopy at an accelerating voltage of 15-25KV and a working distance of 23-26nm.

Results:

See the Appendix IV

(undone) Transmission Electron Microscopy (July 19 to July 20)

Material and methodology:

A coated grid was floated on a droplet of poultry stain of *C. jejuni* or mixture of B_5 CD_{30} and the functionalized nanoparticle on parafilm for 1 minute to permit adsorption of the specimen. The grid was then transferred onto a nearby drop of phosphotungistic acid (PTA) for stain for 30 seconds. Any excess liquid was blotted using a piece of filter paper, and then air-dried for 30 minutes. It was then examined under the Transmission Electron Microscopy. Pictures were taken for future analysis.

1. Exam the poultry strains of B_5CD_6, B_5CD_{30}, Ce0-3, Ce1-9.
2. Exam under the TEM to see if there is any binding between the Poultry bacteria strain B_5 CD_{30} and the functionalized nanoparticle (Cat #, Shelby 6-20-02).
3. Obtain some pictures 07/20/2001.

Results:

Please refer to the Appendix V.

Slide agglutination test:

Materials:

1. Microorganism: Poultry strain B_5CD_{30} of *C. jejuni*, measure $OD_{600nm} = 0.6$
2. Nanoparticles: 250nm functionalized nanoparticles (Cat #, Shelby 6-20-02)
3. Instruments: Microscopy slides, sterilized toothpick, light microscopy.

Material preparation:

1. Microorganism: Poultry strain B_5CD_{30} of *C. jejuni* from liquid culture, centrifuge, PBS buffer suspended, measure $OD_{600nm} = 0.6$,
2. Nanoparticles: serially dilute the nanoparticle solution 1/20, 1/50, 1/100. 1/1000, 1/10000

Experiment:

1. Serially dilute *C. jejuni* to 10^{-5} 10^{-6},
2. Serially dilute functionalized nanoparticles to 1/20, 1/50, 1/100, 1/1000,1/10000.
3. Take one drop of the 10^{-1} concentration of the bacteria on slide.
4. Take one drop of 1/20 conc. of 250nm functionalized nanoparticles on the slides.
5. Use a toothpick to mix the drops.
6. Exam visually unaided for agglutination.
7. Exam under microscopy for agglutination.
8. Repeat the experiment by using different conc. of bacteria (obtained from material preparation) and different proportional nanoparticles (obtained from material preparation).

Results:

No apparent Agglutination in whatever mixture no matter under unaided eyes or under light microscopy.

	1:20	1:50	1:100	1:1000	1:10000
10^{-1}	No	No	No	No	No
10^{-2}	No	No	No	No	No
10^{-3}	No	No	No	No	No
10^{-4}	No	No	No	No	No
10^{-5}	No	No	No	No	No
10^{-6}	No	No	No	No	No

Slide agglutination test

Materials:

1. Buffered poultry strain B_5CD_6 of *C. jejuni*, OD $_{600}$nm=0.6
2. 250nm non-functionalized nanoparticles
3. 250nm functionalized nanoparticles (Cat #, Shelby 6-20-02)

Methods:

1. Serially dilute *C.jejuni* to 10^{-5} 10^{-6},
2. Serially dilute functionalized nanoparticles to 1/10, 1/20, 1/50, 1/100. 1/1000,
3. Mix various conc. of nanoparticles with various conc. of bacteria on the slides.
4. Exam under unaided eyes.
5. Exam under light microscopy 100X, 400X

Results:

No apparent Agglutination in whatever mixture no matter under unaided eyes or under light microscopy.

	1:10	1:20	1:50	1:100	1:1000
10^{-1}	No	No	No	No	No
10^{-2}	No	No	No	No	No
10^{-3}	No	No	No	No	No
10^{-4}	No	No	No	No	No
10^{-5}	No	No	No	No	No
10^{-6}	No	No	No	No	No

Period 5:

In this period of time, got the batch of W.J. P109 (7-17-01) nanoparticles. Mainly tested the effects of this batch of Nanoparticles.

08/27 Page 38

Slide agglutinations

New delivered mannose-nanoparticle W.J. P109 (7-17-01) mix with different conc. of the poultry strains B_5CD_{30}.

Materials:

1. Microorganism: Poultry strain of *C. jejuni* B_5CD_6 from liquid culture, centrifuge, PBS buffer suspended, measure $OD_{600nm} = 0.5$
2. Nanoparticles: functionalized nanoparticles W.J. P109 (7-17-01), serially dilute to 1/10, 1/50, 1/100. 1/1000.
3. Instruments: Microscopy slides, sterilized toothpick, light microscopy.

Experiment:

1. Serially dilute *C. jejuni* to 10^{-3} 10^{-4},
2. Serially dilute functionalized nanoparticles to 1/10, 1/50, 1/100, 1/1000.
3. Take one drop of the original concentration of the bacteria on slide.
4. Take one drop of 1/10 conc. of 250nm functionalized nanoparticles on the slide
5. Use a toothpick to mix the drops.
6. Exam visually by eyes for agglutination.
7. Exam under microscopy for agglutination.
8. Repeat the experiment by using different conc. of bacteria (obtained from material preparation) and different proportional nanoparticles (obtained from material preparation).

	1	1:10	1:50	1:100	1:1000
Original Conc.	No	No	No	No	No
10^{-1}	No	No	No	No	No
10^{-2}	No	No	No	No	No
10^{-3}	No	No	No	No	No
10^{-4}	No	No	No	No	No

08/30

Confocal microscopy

In the morning, confocal microscopy demonstrated. Got the pictures of *C. jejuni* strain of B_5CD_{30} ~~(refer the Appendix III).~~

Results:

Please refer to the appendix VI

C. jejuni colonies photo-taken

Took Pictures of the typical colonies of Poultry stains of *C. jejuni* of Ce0-3, B_5 CD_{30} and keep in file.

Results:

Please refer to the appendix VII.

Period 6:

Got two batches of nanoparticles of Q-26 Pst-mannose and Weijie 8-24-P141 PS-mannose. Did some experiments of Slide agglutination and Viable counting test. Also finished the Growth Curve of Poultry B_5CD_{30}. Tried to find the optimal of concentration of the nanoparticles for which has the maximum of the colony reduction.

Pore size experiment

Materials:

Syringes. Test tubes, 0.2um, 0.45um, 1.0um acrodisces,

Method:

1. Using one syringe, get 1ml of B5CD30 poultry strain.
2. Filter the bacteria by using pre-wetted 0.2um pore size acrodisc. Transfer the solid media, and spread them out. Put into campy-pouch then put into the incubator.
3. Using 0.45um and 1.0um pore size acrodisc, repeat the above experiment procedures
4. Exam the plates after 24 and 48 hours

Results:

exam on Sept.8.				
	0.2um-filtrate	0.45um-filtrate	1.0um-filtrate	no-filter
set 1	no growth	no growth	no growth	little growth
set 2	no growth	no growth	no growth	N/A

exam on Sept.9.				
	0.2um-filtrate	0.45um-filtrate	1.0um-filtrate	no-filter
set 1	no growth	no growth	little growth	obvious growth
set 2	no growth	no growth	little growth	N/A

Sept.15

Pore size experiment (continued, experiment 2)

Try the 1.0um pore size Acrodisc filter. To see what percentage of bacteria come through the filter.

Materials:

1. 1.0um NYL filter (Whatman 1.0um NYL cat. # S6751-2510).
2. 10ml syringe,
3. 5 ml test tubes
4. Poultry strain of B_5 CD_{30} cell suspension, centrifuge, resuspended, buffered to PD=0.3
5. Brucella Broth agar media plates

Procedures:

See Sept. 7th.

Results:

	original	-1	-2	-3
cell	TMTC	TMTC	TMTC	483
filtrate	TMTC	TMTC	TMTC	452

	original	-1	-2	-3
cell	TMTC	TMTC	TMTC	198
filtrate	TMTC	TMTC	TMTC	463

Viable counting

Methods:

1. Prepare bacteria suspension: centrifuge; suspend. PBS-buffered, standardized to OD600nm=0.3
2. Serially dilute to10^{-2} 10^{-3}, 10^{-4}, 10^{-5}
3. Plate them out in duplicate,
4. Take 0.1ml of 10-2 conc. of functionalized nanoparticles (batch #: Q-26, Pst-mannose (EDAC), mix with the different concentration of the bacteria for 30 seconds.
5. Transfer to the BB solid media plate. Using spread plate techniques, plate them out in duplicate.
6. Use the batch of Weijie 08-24-P141 nanoparticles as control 1. Using the same procedures above to plate them out.

Results:

Sept.17. Viable counting. For Q-26.and 08-24-P141 after 48 hours							
	-2	-3		-4		-5	
Q-26	TMTC	TMTC	402	80	72	6	4
08-24-P141	TMTC	TMTC	88	38	40	3	6
Bact.Only	TMTC	TMTC	TMTC	103	97	13	12

Repeat the Sept.17 experiment on Sept 19.

Results:

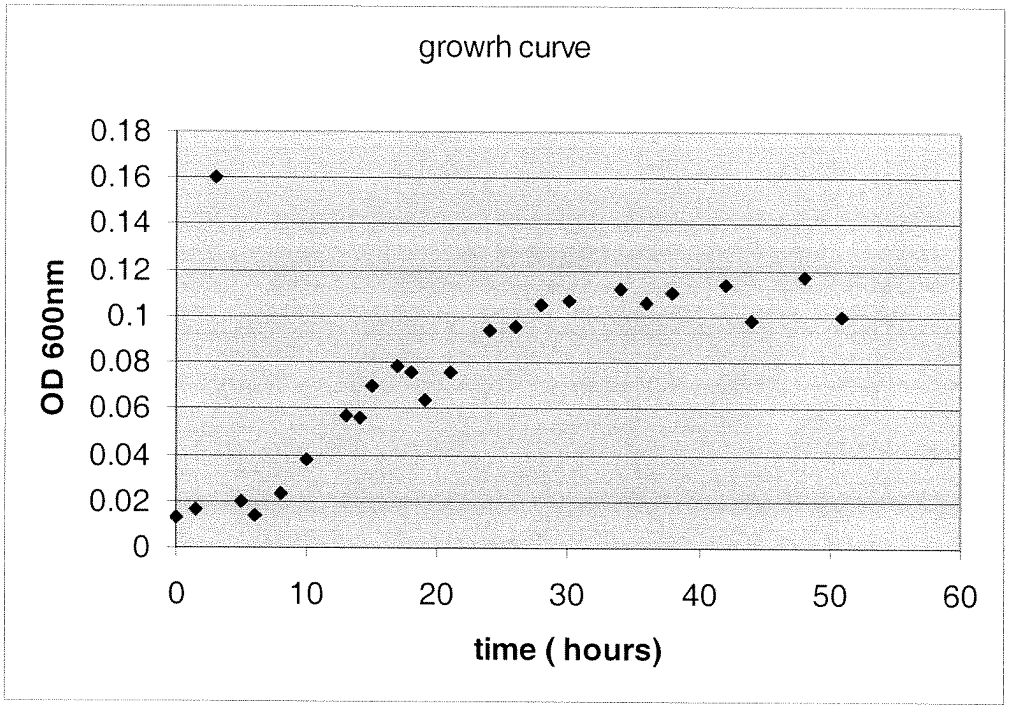
Sept. 19. Viable counting for Q-26 and 08-24-P141.								
	-2	-3	-4		-5		-6	
Q-26	TMTC	TMTC	182	175	12	23	2	0
08-24-P141	TMTC	TMTC	112	121	10	12	1	0
Bact. Only	TMTC	TMTC	165	190	27	15	1	0

Repeat the experiment again on Sept 21.

Sept.24. Viable counting for Q-26 and 08-24-P141.								
	-2	-3	-4		-5		-6	
Q-26	TMTC	TMTC	451	302	50	56	6	8
08-24-P141	TMTC	TMTC	489	TMTC	50	51	3	9
Bact. Only	TMTC	TMTC	TMTC	TMTC	65	92	12	14

Growth Curve of B_5 CD_{30}

About 2 ml Poultry strain of *C. jejuni* B5CD30 grew for 2 days, then inoculated into 120ml Brucella Broth liquid media, then divided into 40 test tubes, each one contained 3ml, then put into the Campy pouch and incubated. Every a few hours, took a test tube and measure the OD600nm against the liquid media as the blank.



Sept 27.

"CFU *versus* Nanoparticle concentration" experiment

Method:

1. Prepare the bacteria: bacteria strain $B_5\,CD_{30}$ cultured for 3 days, centrifuged, suspend. PBS buffered. Standardized to OD_{600}nm=0.3
2. Serially dilute the bacteria to 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6}
3. In duplicate, plate them out on the BB agar plates.
4. Prepare the functionalized nanoparticles Batch Q-26. Dilute the original NP to the conc. of 1/8 X 10^{-2}, 1 /2 X, 1, 2, 4..
5. Prepare the other two batches (weijie 08-24-P141, the PEG-Core-nanoparticle) of nanoparticles the same way.
6. Mix 0.1ml different conc. of the bacteria with 0.1ml different conc. of the nanoparticles for 30 seconds, then transfer to the BB agar plates, spread them out.
7. Seal into the Campy pouch, and put into the incubator.
8. Exam after cultured for 36 and 48 hours.
9.

Results:

	-2		-3		-4		-5		-6	
Bact only	TMTC	TMTC	TMTC	TMTC	167	190	23	15	5	0

Q-26	-2		-3		-4		-5		-6	
1/8x conc.	TMTC	TMTC	TMTC	TMTC	134	148	25	13	2	0
1/4x conc.	TMTC	TMTC	TMTC	TMTC	153	142	13	6	4	1
1/2x conc.	TMTC	TMTC	TMTC	TMTC	167	156	14	26	0	0
10 (-2)	TMTC	TMTC	N/A	TMTC	128	165	10	18	0	3
2x conc.	TMTC	TMTC	TMTC	TMTC	113	N/A	13	16	0	0
4x conc.	TMTC	TMTC	TMTC	TMTC	151	159	7	10	2	1

08/24/p141	-2		-3		-4		-5		-6	
1/8x conc.	TMTC	TMTC	TMTC	TMTC	98	143	21	13	3	1
1/4x conc.	TMTC	TMTC	TMTC	TMTC	139	130	25	7	0	2
1/2x conc.	TMTC	TMTC	TMTC	TMTC	96	103	10	10	0	0
10 (-2)	TMTC	TMTC	TMTC	TMTC	142	121	10	12	N/A	3
2x conc.	TMTC	TMTC	TMTC	TMTC	131	148	23	16	1	0
4x conc.	TMTC	TMTC	TMTC	TMTC	139	152	22	13	2	2

PEG-core	-2		-3		-4		-5		-6	
1/8x conc.	TMTC	TMTC	TMTC	TMTC	0	98	9	N/A	0	0
1/4x conc.	TMTC	TMTC	TMTC	TMTC	64	104	7	3	0	0
1/2x conc.	TMTC	TMTC	TMTC	TMTC	110	96	12	8	1	0
10 (-2)	TMTC	TMTC	TMTC	TMTC	91	114	10	13	0	0
2x conc.	TMTC	TMTC	TMTC	TMTC	89	103	8	7	0	0
4x conc.	TMTC	N/A	TMTC	TMTC	104	96	8	3	0	0

The CFU versus the conc. of the Nanoparticles
(Continued, experiment 2)

Materials:

1. Poultry strain of *Campylobacter jejuni* of B_5 CD_{30}
2. PEG-Core nanoparticles

Prepare the materials:

Poultry strain of *C. jejuni* B_5 CD_{30} cultured in Brucella Broth liquid media for 2 days before use. Centrifuged, Liquid media washed, centrifuged again. Standardized the cell suspension to OD_{600}nm=0.5. Serially diluted to 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6}

PEG-Core nanoparticles (size, about 250-300nm) were synthesized by the Chemistry Department, Clemson University. The original nanoparticles was serially diluted to 1/128 X 10-2, 1/64 X, 1/32 X, 1/16 X, 1/8 X, 1/4 X, 1 X, 4 X, 10 X, 20 X.

Experimental procedures:

1. 0.4ml of liquid media mixed with 0.4ml of various concentrations of the prepared bacteria suspensions for about 30seconds. Took 0.2ml of the mixture, in duplicated, plate them out in Brucella Broth agar plates.
2. 0.4ml of the prepared various concentration of the cell suspensions mixed with 0.4ml various concentrations of the prepared nanoparticle. Took 0.2ml of the mixture, in duplicated, plate them out in Brucella Broth agar plates.
3. Seal into the BBL Campy pouch system and put into the 42-43℃ incubator and cultured for 48 hours before examination.

Results:

	10^{-2}	10^{-3}		10^{-4}		10^{-5}		10^{-6}	
Bact.only	TMTC	TMTC		436	386	40	44	6	4
1/128 X nano	TMTC	TMTC		350	328	28	34	3	2
1/64 X	TMTC	TMTC		322	284	22	33	1	2
1/32 X	TMTC	TMTC		156	278	16	31	1	3
1/16 X	TMTC	TMTC		190	256	20	22	0	2
1/8 X	TMTC	TMTC		145	168	13	18	1	2
1 X 10-2	TMTC	TMTC		112	137	13	8	3	0
4 X	TMTC	TMTC		258	221	23	24	3	2
10 X	TMTC	54	98	34	45	1	2	0	0
20 X	TMTC	240	352	37	19	1	0	0	0

Appendix I Presentation of C.jejuni

Campylobacter jejuni and Nanoparticles

Part 1

Description
of
Campylobacter jejuni

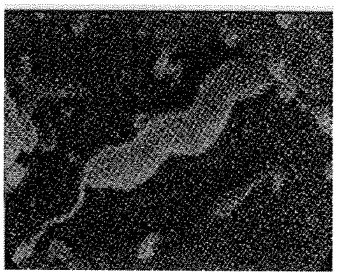
Discovery of *C.jejuni*

Isolated by Dekeyster in 1972 , using a filtration technique.

Description of *C. jejuni*

- 1, Gram negative;
- 2, Curved or spiral rods;
- 3, Size:--------diameter
- 4, Temp: grow between 25-42℃ ;
- 5, Microaerophilic ;
- 6, Does not ferment or oxidize carbohydrates.
- 7, flagella and pilus

An electron micrograph of *C.jejuni*



Culture conditions for *C. jejuni*

- 1, Temperature: can grow between 30-45℃, optimal 42-43℃ ;
- 2, PH: grows well between PH 5.5-8.0; optimal 6.5-7.5; and no growth below PH 4.9
- 3, Oxygen requirement: 5% O_2 ,10% CO_2 and 85% N_2 ;
- 4, Sensitive to drying;
- 5, Sensitive to sodium chloride.[-what-conc.-]

Pathology of *C. jejuni*

- 1, Virulence factors:
  - a, motility;
  - b, adherence; to what-------
  - c, toxins----------
- 2, Diarrhea:
  - a, watery diarrhea (cholera-like);
  - b, bloody diarrhea

Routes of transmission for *C. jejuni*

- 1, Direct contact with infected animals;
- 2, Consumption of untreated water and milk;
- 3, consumption of raw or undercooked meat;

Disease-1:dose and stage of infection

- 1, An infectious dose of as low as 500 bacteria can cause an infection;
- 2, Stages of infection:
  - a, incubation period (from 18hrs to 8 day)
  - b, onset of prodrome
  - c, diarrhea stage (how long it last?)
  - d, recovery stage (how long)

Disease-2: symptoms and diagnosis

- 3, Symptoms:
  - a, fever; (how high)?----
  - b, abdominal cramping / pains;
  - c, diarrhea (with or without blood or fecal leukocytes);
  - d, headache; vomiting etc;
- 4, Diagnosis: symptoms and bacteriology/ serodiagnosis / endoscopy. Diagnosis can be made only by detecting *C. jejuni* in the feces

Disease-3: complications

- 1, Intestinal complications:
  - a, hemorrhage
  - b, stomach ulceration
  - c, perirectal abscess
- 2, Extraintestinal complications: bacteremia;
- 3, Infection of hepatobiliary system;
- 4, Renal and urinary tract disease;
- 5, Late-onset syndrome: -?--G-B syndrome.

Disease-4: treatment

- 1, *C. jejuni* are susceptible to a variety of antibiotics including erythromycin and fluoroquinoline such as-----
- 2, Indication: those patients having more than 8 stools / day, bloody diarrhea, high fever or symptoms persisting for > 1 week.
- 3, Duration of treatment: 5--7 days .

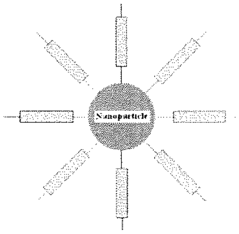
Epidemiology

- 1, Age : affect all age groups, and in 1998, the incidence was 530/100,000 for children younger than 1 year old. Young adults (15-44 years) show another peak.
- 2, Sex: incidences in male were 30% higher than female.
- 3, Seasonal distribution: generally, a peak incidence in the warm months of the years.
- 4, Trend: many developed countries have experienced an increase in the number reported.

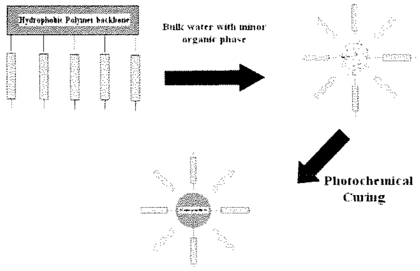
Part 2

The nanoparticles

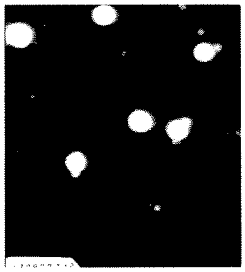
Nanoparticle-1



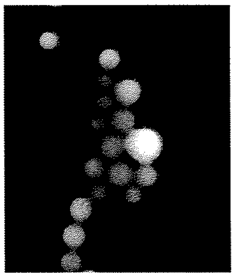
Nanoparticle-2 (synthesis)


Hydrophobic Polymer backbone
Bulk water with minor organic phase
Photochemical Curing

Nanoparticle-3 (Nano-core)



Nanoparticle-4 (Nano-core PEG)



How do nanoparticles work?--1

- The nanoparticles consist of hydrophobicic core polymer chains covalently linked to hydrophilic molecules which are known host surface ligands (e.g. fibronectin fragments and α -D-galactopyranosyl- β - D-galactopyranoside) with high affinity for bacterial adhesins.

How do nanoparticles work?--2

- Since there are bioactive molecules in the surface of the nanoparticles, the nanoparticles should effectively compete with the mucosal tissues of the host for microbial attachment.
- Because the nanoparticles contain a large surface density of functional sites, they should be able to agglutinate large numbers of pathogens, prevent them from binding to the host, and enable them to be safely washed from the body.

Part 3

The experiments

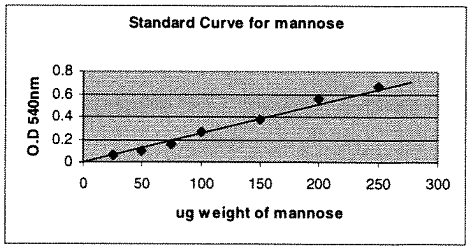
Experiment 1: (protocol) Reducing sugar determination by the Bernfeld method

- 1. 1 ml of Bernfeld reagent to a series of test tubes, add 0.5 ml dH_2O to each test tube.
- 2. Add 0.5 ml of sample to the tubes.
- 3. To establish a mannose standard curve, add 0.5 ml of varying conc. of mannose to a series of tubes.
- 4. Add 0.5 ml of H_2O to one tube as blank.
- 4. Boil all tubes for 10 min, then cool and add 1 ml of H_2O.
- 5. Read O.D 540nm for all tubes against the blank.
- 6. Calculate glucose equivalents in each sample.

The mannose standard curve-1

ul.1mg/ml	dH2O	ug	O.D	O.D	ave. O.D
0	500	0	0	0	0
25	475	25	0.089	0.036	0.0625
50	450	50	0.116	0.074	0.095
75	425	75	0.154	0.15	0.152
100	400	100	0.29	0.244	0.267
150	350	150	0.379	0.367	0.373
200	300	200	0.587	0.533	0.56
250	250	250	0.681	0.661	0.671

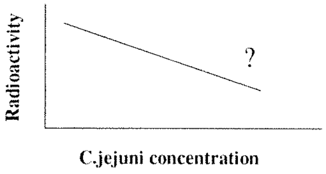
Mannose standard curve-2



Experiment 2: The reduction of Radioactivity

- 1. Use radioisotope labeled mannose (^{3}H or ^{14}C).
- 2. Mix the various concentration of *C. jejuni* sample together with radio-labeled mannose.
- 3. Filter the samples, then measure the radioactivity of each sample.
- 4. Since mannose will bind with the *C. jejuni* and aggregate, then can not pass through the filter. The radioactivity will decrease with the increasing concentration of *C. jejuni*.

Expected result for radio-labeled mannose



Experiment 3: Nanoparticle Slide Agglutination

- 1, Different proportions of bioactive nanoparticle suspensions (250 nm and 750 nm) will be prepared and mixed with various conc. of *C. jejuni* on a microscope slide.
- 2, Nonfunctionalized nanoparticles with PEG side-chains will be used as controls.
- 3, put soluble mannose will reduce the aggregration of C. and .l.........(serve as another control)
- 3, The slides will be observed over a oblique light (visually unaided and also 450 X, high dry) for apparent agglutination.

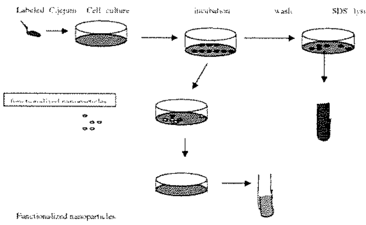
Experiment 4: Spread plate count

- 1, 50 ml of an early stationary phase culture of *C. jejuni* will be mixed with various compositions of synthesized nanoparticles.
- 2,The mixture will be stirred at 50 rpm for 5 min at RT.
- 3, Serial dilutions of each mixture in peptone water will be plated in duplicate on a selective agar medium.
- 4,Cell suspensions at the same conc. (having no nanoparticles) will serve as controls.
- 5,Plates will be incubated under optimal conditions.
- 6,Reduction in colony-forming units (CFU) will be recorded.

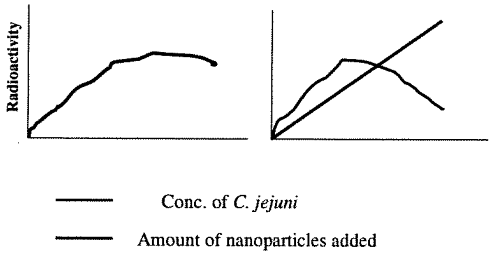
Experiment 5: Protocol for SEM

- **1. Fixation: using 3.5% cacodylate buffered Glutaradehyde at pH 7.2 for 24 hrs at 4°C**
- **2. Dehydration: after removal from the fixative , samples will be dehydrated in ETOH with increasing conc. (50%,70%, 85%, 95% for 15min each and 100% for two 30min intervals.**
- **3. Critical point dry of the samples**
- **4. Mount the samples on Aluminum stubs.**
- **5. Coat mounted stubs with gold to about 5 nm.**
- **6. Exam the samples under Scanning Electron Microscopy.**

Experiment 6: C.jejuni grown in Brucella broth supplemented with sodium acetate



Expected result



Experiment 1: (protocol) Reducing sugar determination by the Bernfeld method

- 1. 1 ml of Bernfeld reagent to a series of test tubes, add 0.5 ml dH_2O to each test tube.
- 2. Add 0.5 ml of sample to the tubes.
- 3. To establish a mannose standard curve, add 0.5 ml of varying conc. of mannose to a series of tubes.
- 4. Add 0.5 ml of H_2O to one tube as blank.
- 4. Boil all tubes for 10 min, then cool and add 1 ml of H_2O.
- 5. Read O.D 540nm for all tubes against the blank.
- 6. Calculate glucose equivalents in each sample.

Experiment 2: The reduction of Radioactivity

- 1. Use radioisotope labeled mannose (^3H or ^{14}C).
- 2. Mix the various concentration of *C. jejuni* sample together with radio-labeled mannose.
- 3. Filter the samples, then measure the radioactivity of each sample.
- 4. Since mannose will bind with the *C. jejuni* and aggregate, then can not pass through the filter. The radioactivity will decrease with the increasing concentration of *C. jejuni*.

Experiment 3: Nanoparticle Slide Agglutination

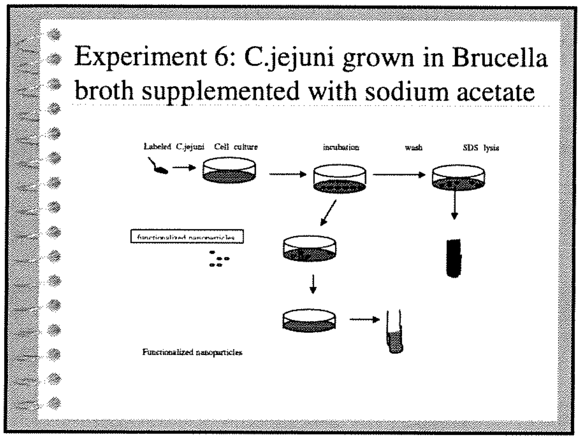
- 1, Different proportions of bioactive nanoparticle suspensions (250 nm and 750 nm) will be prepared and mixed with various conc. of *C. jejuni* on a microscope slide.
- 2, Nonfunctionalized nanoparticles with PEG side-chains will be used as controls.
- 3, put soluble mannose will reduce the aggregration of C. and .l.........(serve as another control)
- 3, The slides will be observed over a oblique light (visually unaided and also 450 X, high dry) for apparent agglutination.

Experiment 4: Spread plate count

- 1, 50 ml of an early stationary phase culture of *C. jejuni* will be mixed with various compositions of synthesized nanoparticles.
- 2,The mixture will be stirred at 50 rpm for 5 min at RT.
- 3, Serial dilutions of each mixture in peptone water will be plated in duplicate on a selective agar medium.
- 4,Cell suspensions at the same conc. (having no nanoparticles) will serve as controls.
- 5,Plates will be incubated under optimal conditions.
- 6,Reduction in colony-forming units (CFU) will be recorded.

Experiment 5: Protocol for SEM

- **1. Fixation: using 3.5% cacodylate buffered Glutaradehyde at pH 7.2 for 24 hrs at 4°C**
- **2. Dehydration: after removal from the fixative , samples will be dehydrated in ETOH with increasing conc. (50%,70%, 85%, 95% for 15min each and 100% for two 30min intervals.**
- **3. Critical point dry of the samples**
- **4. Mount the samples on Aluminum stubs.**
- **5. Coat mounted stubs with gold to about 5 nm.**
- **6. Exam the samples under Scanning Electron Microscopy.**



Experiment 6: C.jejuni grown in Brucella broth supplemented with sodium acetate

Appendix II : email

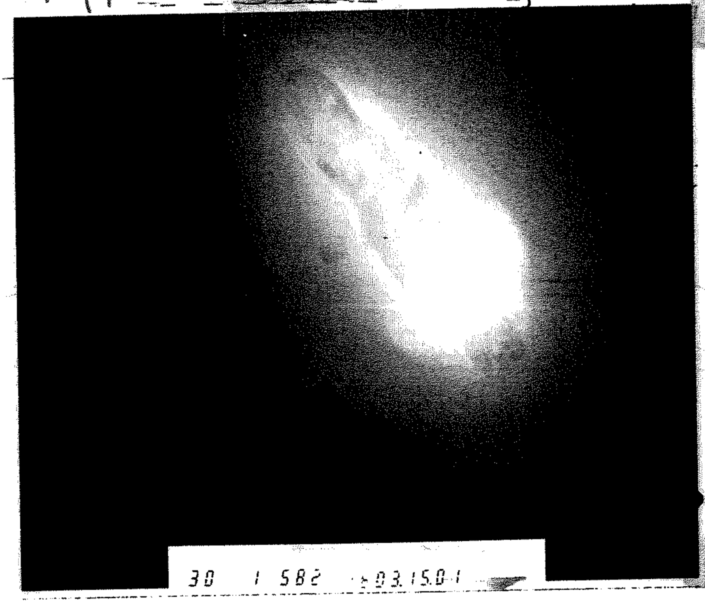
Subject: [Fwd: Campylobacter]
Date: Thursday, January 25, 2001 10:42 AM
From: Fred Stutzenberger <sfred@CLEMSON.EDU>
To: <tzuenrt>

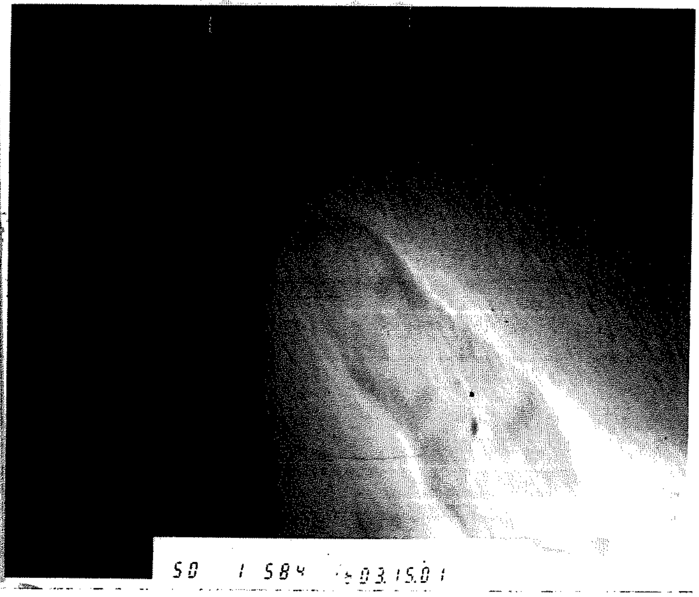
```
>X-Time: <200101251433.f0PEX6O27997>
>Date: Thu, 25 Jan 2001 09:32:49 -0500
>From: Jesse Grimes <jesse_grimes@ncsu.edu>
>X-Accept-Language: en
>MIME-Version: 1.0
>To: sfred@CLEMSON.EDU
>Subject: [Fwd: Campylobacter]
>
>Fred:
>Here is a response from Brian on the campy issue.
>Sotgun news: I have placed some cardboard spacers between the stock and
>but plate on my Ithaca double to create alomost a 14 1/2" LOP. I can
>also take one out and have the LOP pretty close to 14 3/8". I plan to
>shoot it some this weekend and also pattern both barrels. I will let you
>know how it turns out.
>Thanks,
>Jesse
>
>-------- Original Message --------
>Subject: Re: [Fwd: Campylobacter?]
>Resent-Date: Thu, 25 Jan 2001 9:08:01 est5edt
>Resent-From: Jgrimes.poultry.CALS.NCSU@poultry.poulsci.ncsu.edu
>Resent-To: jgrimes@unity.ncsu.edu
>Date: Thu, 25 Jan 2001 09:07:18 EST5EDT
>From: "Brian Sheldon" <bsheldon@poultry.poulsci.ncsu.edu>
>Organization: NCSU Department of Poultry Science
>To: Jesse Grimes <jesse_grimes@ncsu.edu>
>
>Jesse:
>
>Campylobacter should not be growing under aerobic conditions (i.e.
>21% O2) and thus I would question if in fact the organisms are
>campylobacter.  They need only small amounts of O2 of around 3-6%.
>I would suggest that they continue their confirmation studies to make
>sure that they have Campy and not some contaminants.  I have never
>used the campy pouches but have purged a bag or anaerobe jar
>containing the plates with a gas mixture of (5% O2/10% CO2/85% N2).
>Thus, I cannot respond to how well they grow under the pouch
>conditions.  I assume they are using a commercial product that should
>produce the right gas mixture for culturing Campy.  Using Phase
>contrast or dark filed microscopy they should be able to see the
>typical spiral curved rod shape for young cells but late stationary
>phase cells may be coccoid shaped.
>
>Campy may produce very different colony characteristics depending
>on the media used for culturing, incubation temperature, and if the
>inoculations were made from a broth system versus a solid media.
>Since they did not indicate what media they had used I cannot
>comment on the colony characteristics.  On a Campy agar base
>medium, Campy may appear flat and gray with irregular edges or it can
```

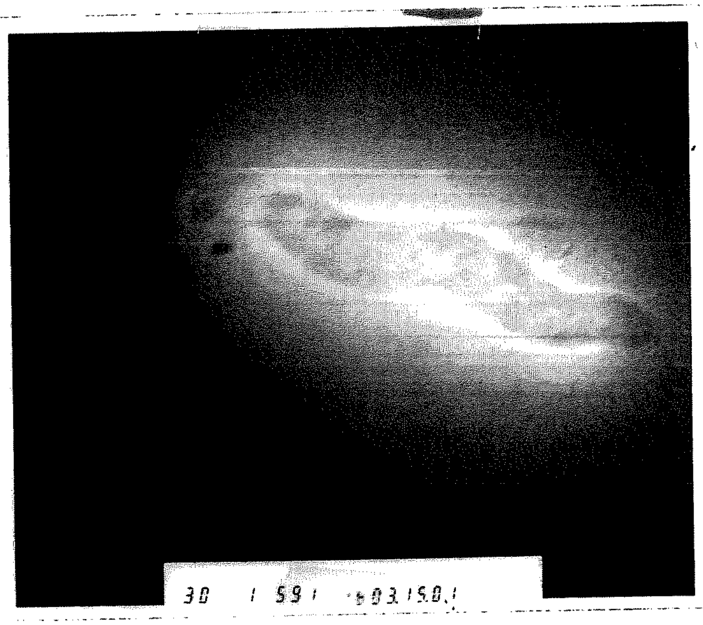
>also appear raised and round with a mucoid appearance. If the surface
>of the media is slightly wet Campy can also produce a smearing
>colony that spreads across the plate.
>
>I suggest that they request the colony characteristics from Difco for
>the Campy agar with supplements and see if they match what they are
>observing in the lab. Campy can be rather difficult to grow, especially
>if you are not use to working with the organism. Furthermore, they
>may be difficult to revive from a storage state. I would recommend
>that they make several transfers of the strains over several
>generations in a liquid media (i.e. Brucella broth) or Campy enrichment
>broth before transferring to a solid media. They should also think
>about making a wet mount of the culture to see if they see the
>characteristic darting motility feature of this organism plus other
>rconfirmation techniques as needed.
>
>Since the nature of this study is to produce these nanoparticles that
>have an affinity for Campy, it is imperative that they confirm that they
>actually have Campy. If there is any question another possibility
>would be for them to purchase a fresh Campy jejuni strain from the
>American Type Culture Collection (ATCC). However, the strains that
>we sent should be Campy strains unless they have become
>contaminated. I hope this helps.
>
>Brian
>
>
>
>Date sent: Wed, 24 Jan 2001 16:36:00 -0500
>From: Jesse Grimes <jesse_grimes@ncsu.edu>
>To: brian_sheldon@ncsu.edu
>Subject: [Fwd: Campylobacter?]
>
>Brian:
>Any comments on the campi?
>Thanks,
>Jesse
>
>-------- Original Message --------
>Subject: Campylobacter?
>Resent-Date: Wed, 24 Jan 2001 14:13:06 est5edt
>Resent-From: Jgrimes.poultry.CALS.NCSU@poultry.poulsci.ncsu.edu
>Resent-To: jgrimes@unity.ncsu.edu
>Date: Wed, 24 Jan 2001 14:12:50 -0500
>From: Fred Stutzenberger <sfred@CLEMSON.EDU>
>To: jesse_grimes@ncsu.edu
>
>>X-Time: <200101241522.f0OFMA027873>
>>User-Agent: Microsoft-Entourage/9.0.2509
>>Date: Wed, 24 Jan 2001 10:22:11 -0500
>>Subject: Campylobacter?
>>From: Tzuen-Rong Tzeng <tzuenrt@CLEMSON.EDU>
>>To: Fred Stutzenberger <sfred@CLEMSON.EDU>
>>Mime-version: 1.0
>>
>>Jesse,

>>Regarding the Campylobacter, when grown in Campy Pouch at 42°C the colonies
>>are small and rough. However, they DO grow will outside of the pouch at
>>42°C. The colonies are larger and smooth. I wander if these are typical
>>colony morphology. We are in the process of verifying the strains on DIFCO
>>Campylobacter Agar with supplements. Ask Brian what he thinks about this.
>>
>> Best Regards,
>>
>>Fred
>>
>>
>>
>>
>Dr. Brian W. Sheldon
>Department Extension Leader & Professor of Poultry Science
>234D Scott Hall
>Box 7608
>North Carolina State University
>Raleigh, NC 27695-7608
>(919) 515-5407 FAX: (919) 515-7070
>Brian_Sheldon@ncsu.edu (email)
>

Appendix III TEM of ATCC Strain of C.jejuni

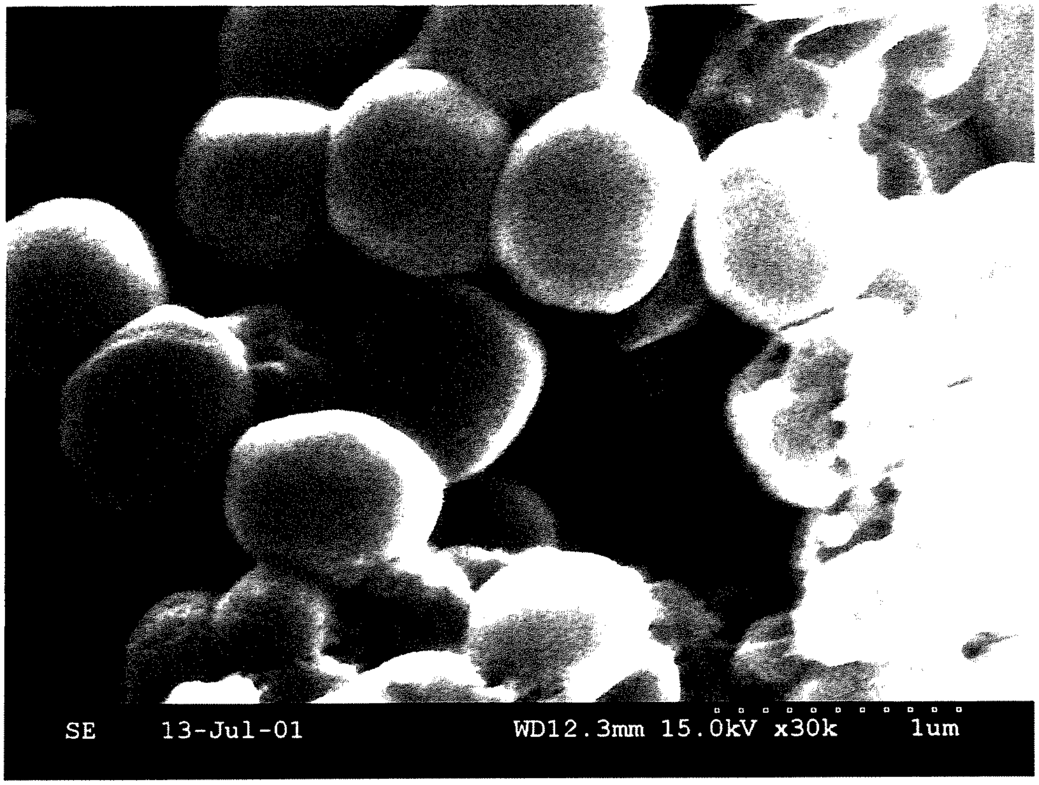






ATCC

Appendix IV SEM.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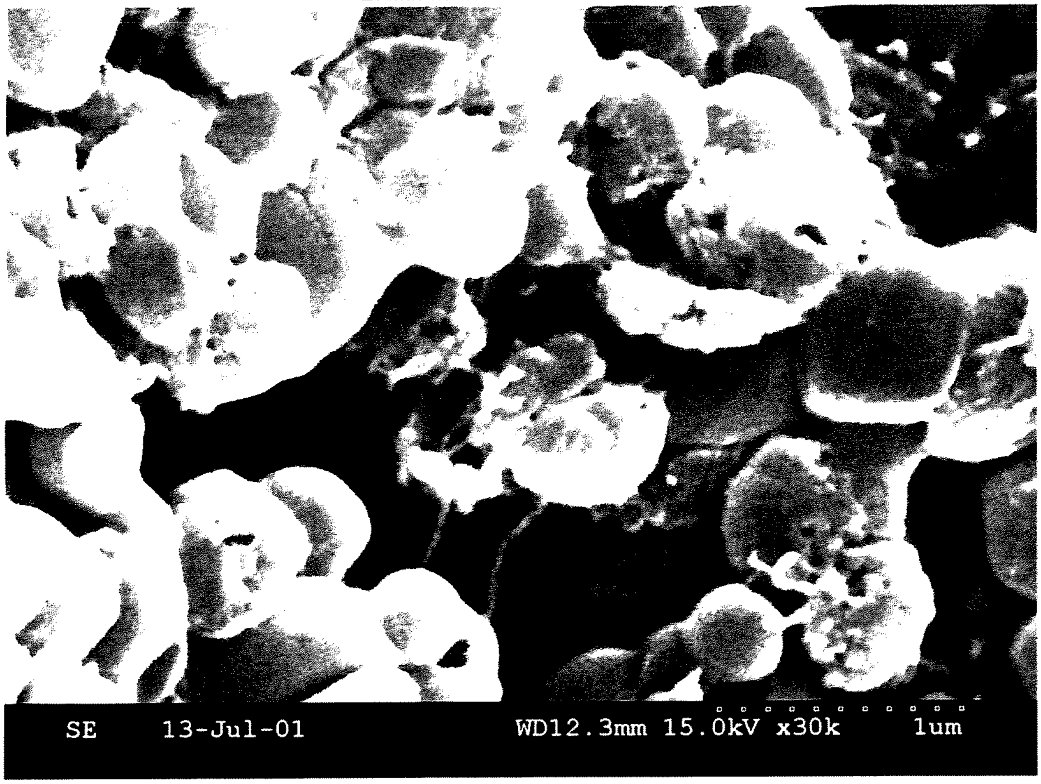
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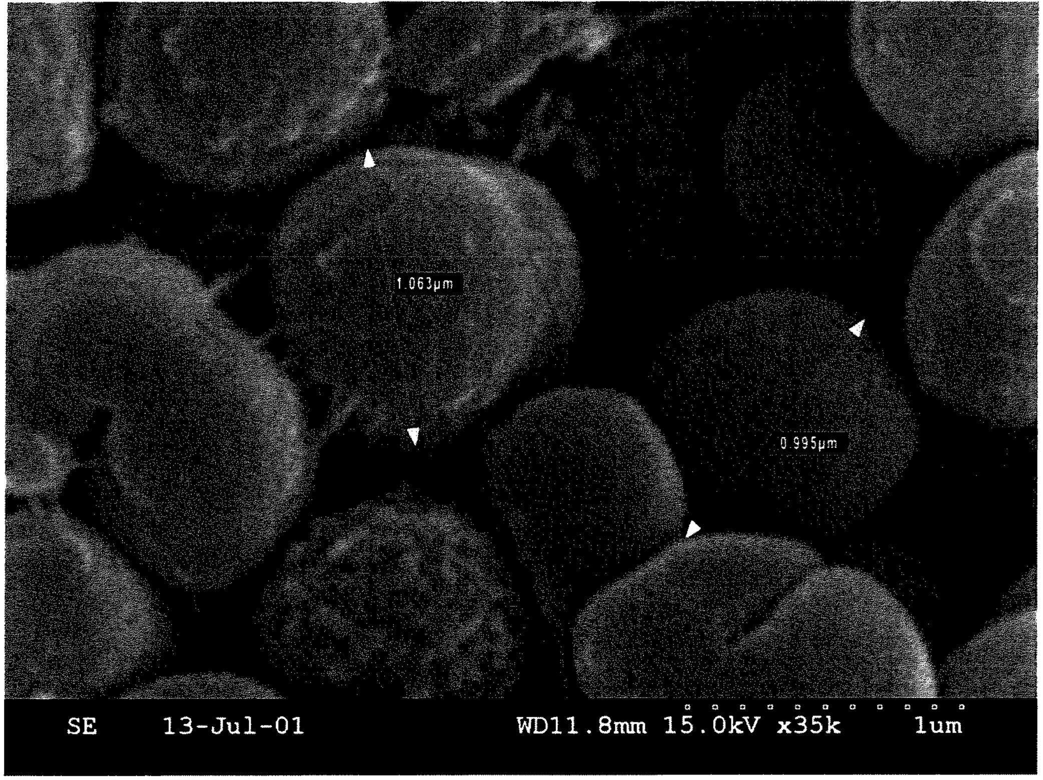
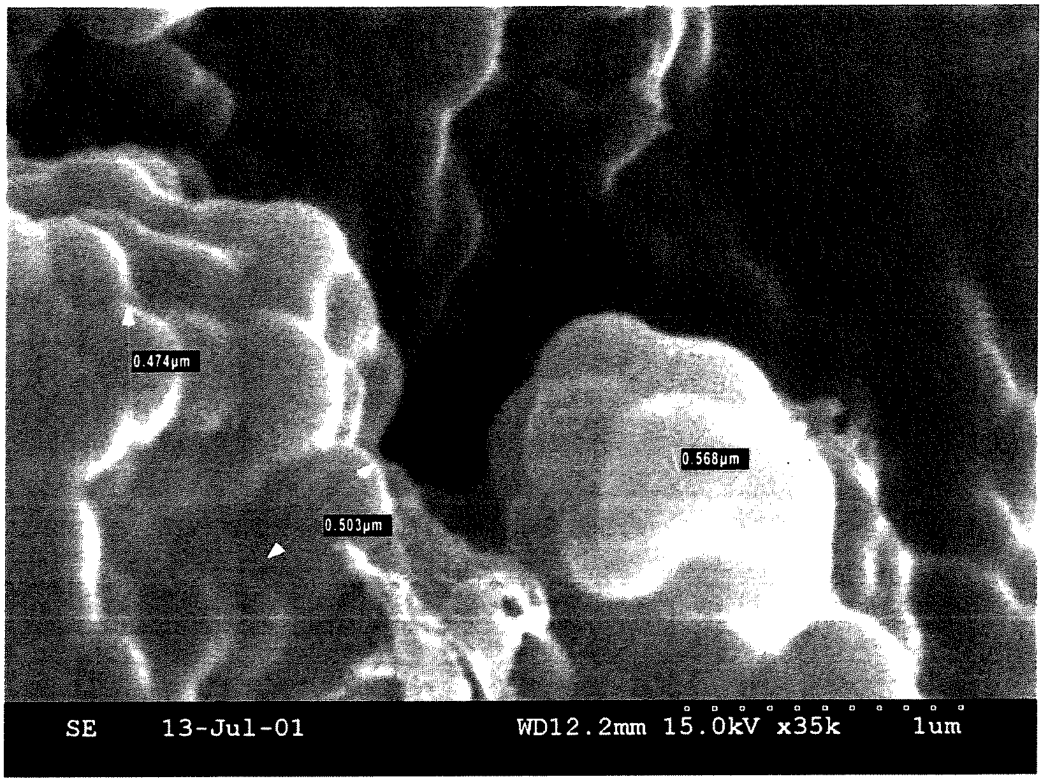
E:\bact-lyphi..PCI

E:\bact-storageonly.PCI

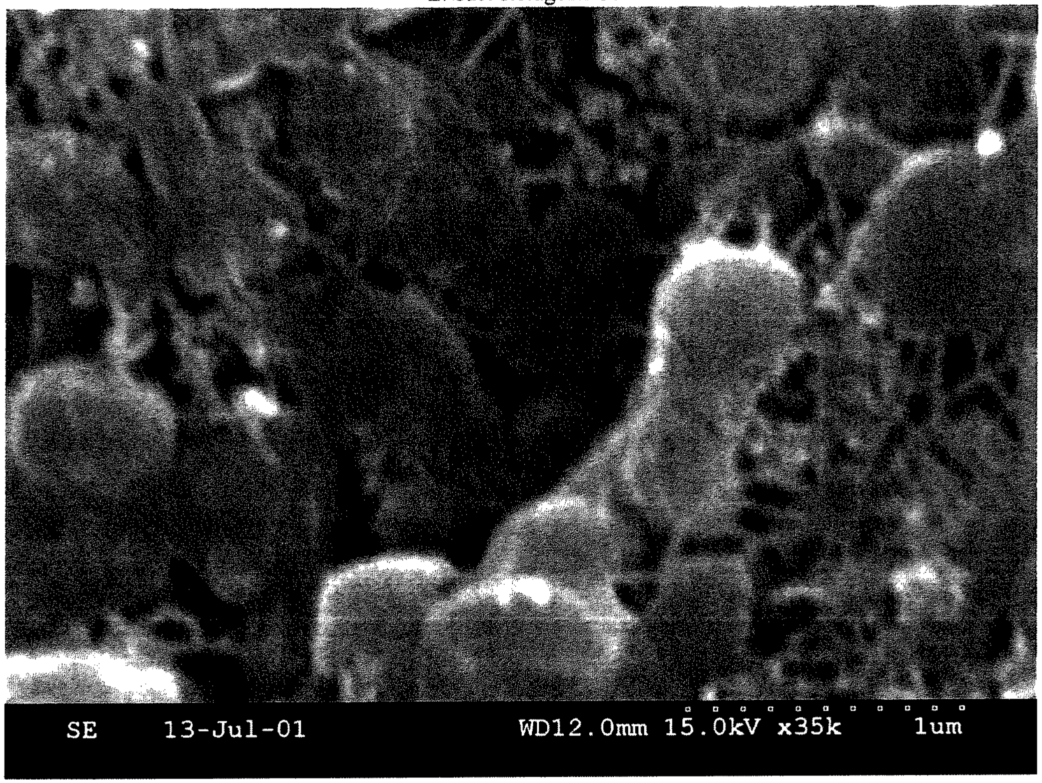


E:\nonfunnano.PC1

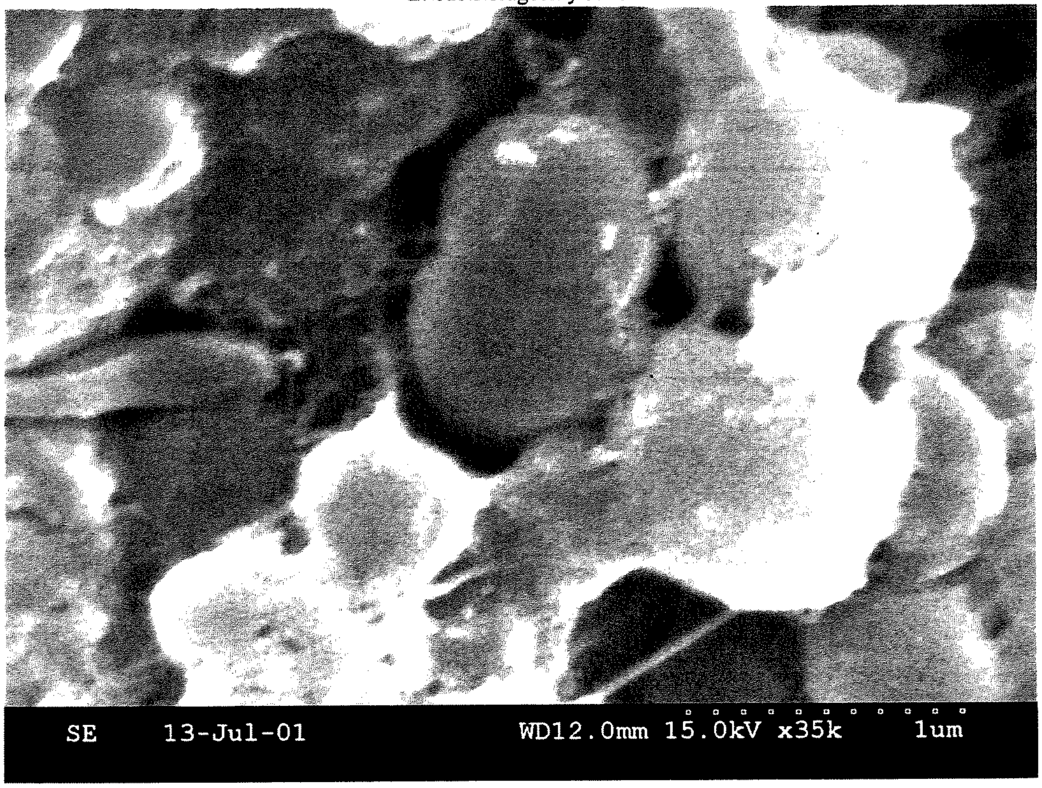


Y

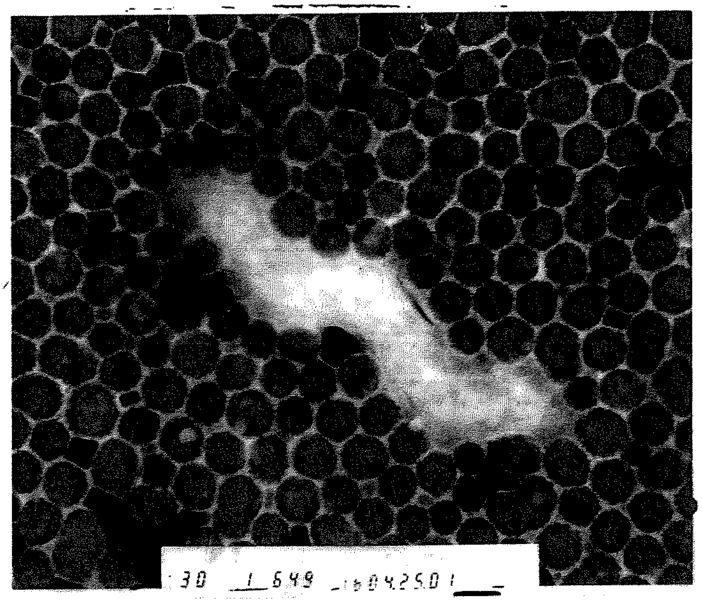
E:\bact-storage2.PCI

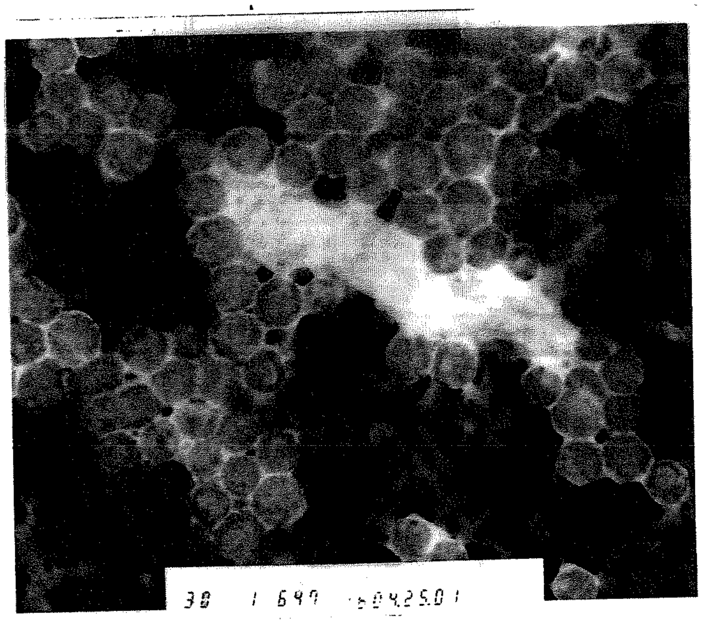


E:\bactstorageonly 3.PCI

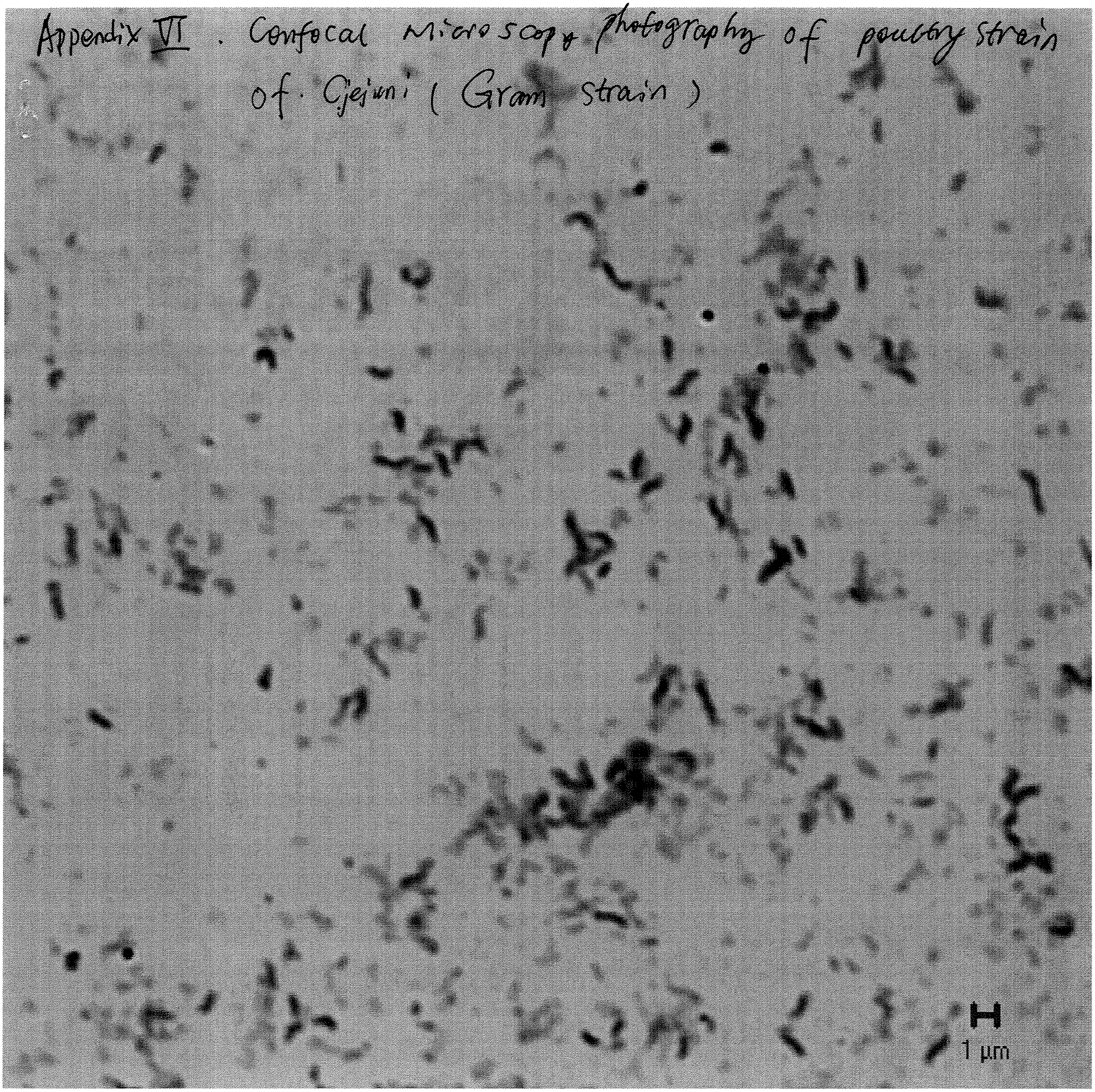


Appendix V TEM of poultry strain & nanoparticles

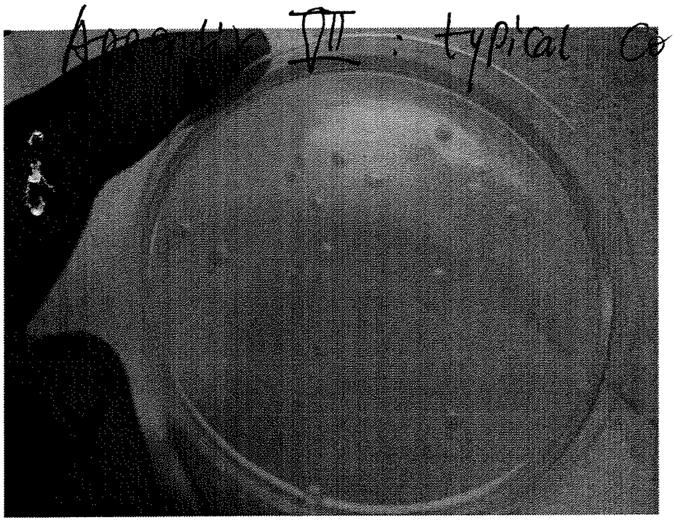


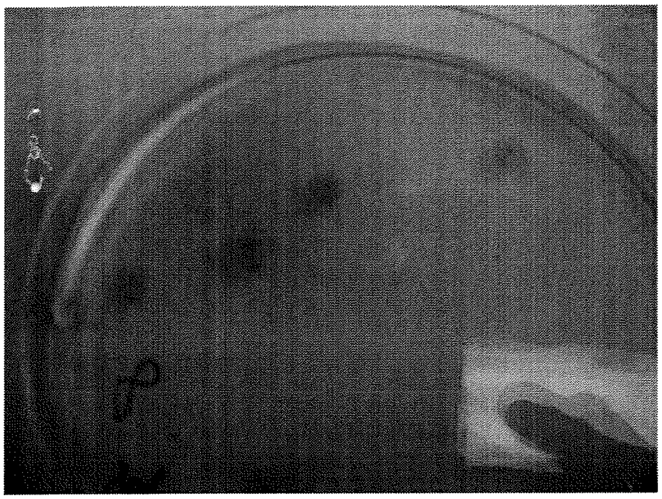


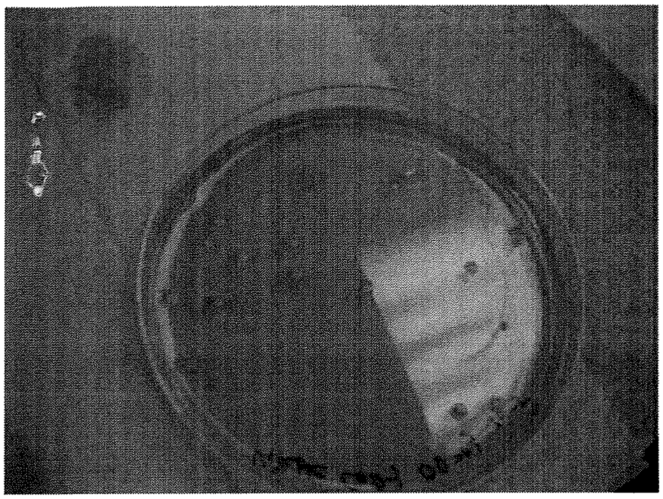
Appendix VI . Confocal Microscopy photography of poultry strain of C. jejuni (Gram strain)



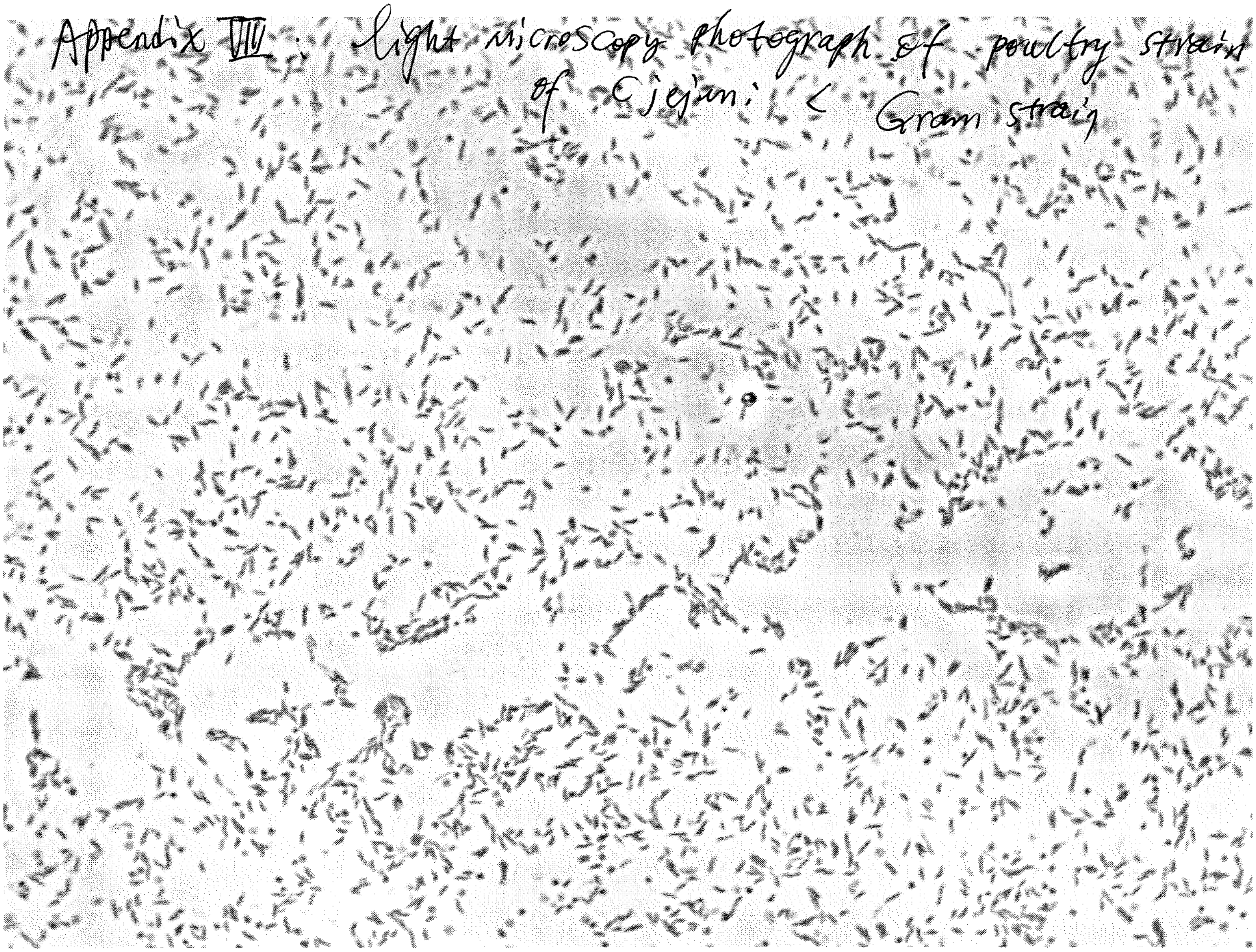
Appendix VII : typical colonies of poultry strain of C. jejuni




P



Appendix VIII : light microscopy photograph of poultry strain of C jejuni < Gram strain



APPENDIX I

TO: Stephen R. Chapman
FROM: Clinton Barry CB
FILE: Stutzenberger
RE: Patentability of Biofunctionalized Nanoparticles
DATE: July 10, 2002

Question Posed: Is the development of biofunctionalized nanoparticles capable of determining adhesive affinities of pathogenic bacteria patentable over prior art?

Brief Answer: Probably, yes. Although nanoparticle technology has yielded particles capable of drug delivery of specific recognition sites, the biofunctionalized nanoparticles as well as the methods to produce and utilize them appear to be unique and non-obvious in view of current art.

ARGUMENT

Patentable subject matter must be identified as being in at least one of four categories: process, machine, manufacture, or composition of matter. 35USC 101. The biofunctionalized nanoparticles described by Dr. Fred Stutzenberger clearly satisfy the criteria as a manufacture and as a method or process.

Patentability also requires that the invention be useful, unique, and non-obvious. (35USC 101, 103) The disclosed invention clearly satisfies the utility requirement considering the potential for drug delivery. Non-obviousness and uniqueness must be evaluated in current technology, either ongoing works or published works. 35USC 102 A &B, 103.

UNIQUENESS

A patent search was conducted in order to evaluate the uniqueness of the invention. From the U.S. Patent and Trademark Office website, "nanoparticles", "bacterial", and "adhesin(s)" were used as keywords in the search. The search yielded results that demonstrated the uniqueness of the biofunctionalized nanoparticles. Among the results, five patents contained similarities to the invention. Those five patents, along with their patent numbers, are listed below. Each patent name is followed by a brief summary and a comparison to the proposed invention.

6,387,408: Adhesive drug delivery composition
6,355,276: Adhesive drug delivery composition

United States Patents 6,387,408 and 6,355,276 are methods that apply "adhesive materials from the fimbriae of bacteria or synthetic analogues or fragments combined with a drug to provide for attachment to the gut of a mammal". In comparison to the disclosed invention, the two patents use adhesives and drug delivery, but neither includes the use of biofunctionalized nanoparticles.

6,291,435: Treatment of diarrhea caused by enteropathogenic Escherichia coli

U.S. Patent 6,291,435 "relates to compositions and methods useful to treat diarrhea and related conditions". This patent is similar to the disclosed invention because it uses a method that targets drugs to bacteria, but this patent deals solely with the treatment of diarrhea and does not include the use of biofunctionalized nanoparticles.

6,040,421: Escherichia coli O157:H7 epithelial adhesion and vaccine
5,798,260: Escherichia coli O157:H7 epithelial adhesin

U.S. Patents 6,040,421 and 5,798,260 are similar to the disclosed invention because of the mention of bacterial adhesins, but these two patents use "a continuous segment of chromosomal DNA that encodes an adhesin that medicates bacteria", a method which the disclosed invention does not apply.

CONCLUSION

The disclosed invention satisfies patentability requirements as a manufacture and as a method or process. The invention has the possibility to be extremely useful, and the results of the patent search demonstrated its uniqueness and non-obviousness. Following the research and through discussions with Dr. Stutzenberger, we believe that the invention satisfies the requirements for patentability.